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REMARKS

The Applicant deeply appreciates the indication that Claim 25 is allowed. Also, the Applicant deeply appreciates the indication that the prior rejection under 35 U.S.C. Section 112, second paragraph, of Claims 1-9, 23-24, 25, 28, 34 and 36 has been withdrawn.

Rejection under 35 U.S.C. Section 112:

Claims 29 and 30 were rejected under 35 U.S.C. Section 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. These Claims were objected to as being broader than Claim 26 from which they depend. Claim 26 is now cancelled. Therefore, it is respectfully believed that this rejection is rendered moot. Therefore, Claims 29 and 30 overcome the rejection under 35 U.S.C. Section 112, second paragraph.

Claims 3-6, 12-14, 24, 34 and 36 were rejected under 35 U.S.C. Section 112, first paragraph, as being indefinite for not reasonable providing enablement commensurate with the scope of the claimed invention. In particular, it is held that the specification does not enable any person skilled in the art to which it pertains, or with it most nearly connected, to make and use the invention commensurate in scope with the claims. It is respectfully believed that under the Manual for Patent Examining Procedure (M.P.E.P.) Section 2164.01, the test of enablement is any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and

use the claimed invention. The standard for determining whether the specification meets the enablement requirement was cast in the Supreme Court decision of Mineral Separation v. Hyde, 242 U.S. 261, 270 (1916) which postured the question: is the experimentation needed to practice the invention undue or unreasonable? That standard is still the one to be applied. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Accordingly, even though the statute does not use the term "undue experimentation," it has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). See also United States v. Telectronics, Inc., 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988).

Applicant is providing: a first article, which is attached in Appendix A, KWEKKEBOOM, ET AL., *Cholecystokinin Receptor Imaging Using An Octapeptide DTPA-CCK Analogue In Patients With Medullary Thyroid Carcinoma*, European Journal of Nuclear Medicine, 2000, Vol. 27, pp. 1312-1317; a second article, which is attached in Appendix B, REUBI, ET AL., *Unsulfated DTPA- And DOTA-CCK Analogs As Specific High-Affinity Ligands For CCK-B Receptor-Expressing Human And Rat Tissues In Vitro And In Vivo*, European Journal of Nuclear Medicine, 1998, Vol. 25, pp. 481-490; and a third article, which is attached as Appendix C, M DE JONG, ET AL., "*Preclinical And Initial Clinical Evaluation Of 111In-Labeled Nonsulfated CCK8 Analog: A Peptide For CCK-B Receptor-Targeted Scintigraphy And Radionuclide Therapy,*" Journal of Nuclear Medicine, Vol. 40, Issue 12 2081-2087, 1999, Society of Nuclear Medicine. These Articles demonstrate that there is a good correlation

between in vitro and in vivo experiments obtained both with radioiodinated CCK-peptides and with radiolabelled metal chelated CCK- peptides. Furthermore, a person skilled in the art would easily ascertain that good diagnostic results are the direct result of the receptor-specific uptake of the labelled peptide by the tumour in question, so that a medicament with favorable therapeutic properties can be obtained (if applied in the correct dosages) by substitution of the label (radioisotope) without undue or unreasonable experimentation. In other words, substitution of one radioisotope by another suitable for therapy cannot change its receptor-specific behavior and therefore no undue experimentation is necessary. This is even after attaching a (metal-containing) chelating group.

Therefore, the rejection of Claims 3-6, 12-14, 24, 34 and 36 under 35 U.S.C. Section 112, first paragraph, is overcome.

Rejection under 35 U.S.C. Section 103(a):

Claims 1, 2, 4-7, 12, 26, 29, 30, 34 and 35 were rejected under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. Claims 1, 2, 3 and 12 are now amended to recite:"...CCK-B receptor expressing tumours selected from the group consisting of Small Cell Lung Carcinoma, Medullary Thyroid Carcinoma, Breast Carcinoma, Stromal Ovarian Carcinoma and Muscle Sarcoma..." in lieu of "malignant tumours." This terminology is found in Claim 3 and now cancelled Claims 4 and 5. No new matter has been added.

It is respectfully believed that the experiments described by Slaninova et al. have nothing

to do with the intended use of the pharmaceutical compositions as claimed in the present application, because Slaninova et al. describe only a tool for in vitro testing of CCK compounds. Nowhere in Slaninova et al. the preparation of a ligand suitable for human use has been suggested, let alone a pharmaceutical composition comprising such a ligand for cancer application. Therefore, combining Slaninova with Sethi et al. or with other related publications, wherein the expression of CCK-B receptors in a special cancer type, viz, small cell lung cancer (SCLC), is described, would be merely artificial (hindsight reasoning) in "explaining" the claimed pharmaceutical composition for treating human tumors, or, in other words, it would not be obvious for a person with ordinary skill in the art to associate these documents with each other much less the association of four documents with each other in order to arrive at such a composition.

It is respectfully believed that it is improper to apply an "obviousness to try" standard or indulge in hindsight evaluation or reconstruction. See Ecolochem, Inc. v. Southern California Edison Co., 56 U.S.P.Q.2d 1065 (Fed. Cir. 2000). In this case, what is obvious is that which can only be deduced by a logical step-by-step reasoning process from the premises furnished by the prior art. There is no logical step-by-step reasoning process that can be developed from the premises furnished by the prior art and a showing of a suggestion, teaching or motivation to combine the prior art references is an essential component of an obviousness holding. C. R. Bard, Inc. v. M3 Systems, Inc., 48 U.S.P.Q.2d 1225, 1232 (Fed. Cir. 1998).

Therefore, Claims 1, 2, 3 and 12 overcome the rejection under 35 U.S.C. Section 103(a)

over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. Claim 26 is hereby deleted and it is respectfully believed that this rejection is rendered moot. Since Claims 6, 7, 34 and 35 depend from and contain all of the limitations of Claims 1, 2 and 3, as amended, then Claims 6, 7, 34 and 35 overcome the rejection under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. in the same manner as Claims 1, 2, 3 and 12 listed above.

Claims 29 and 30 are rejected under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. These Claims are now placed in independent form and include all of the limitations of cancelled Claim 26. Both Claims 29 and 30 are restricted to Diethylenetriaminepentaacetate (DTPA). It is believed that DTPA does not appear in the cited References. Therefore, it is respectfully believed that all claim limitations must be considered. In re Fine, 837 F.2d 1071, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988).

Therefore, Claims 29 and 30 overcome the rejection under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al.

Therefore, Claims 1, 2, 6, 7, 12, 29, 30, 34 and 35 overcome the rejection under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al.

Claims 1, 2, 4-9, 12-14, 23 and 26-35 were rejected under 35 U.S.C. Section 103(a) over

Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. and further in view of McBride et al. (United States Patent No. 5,620,675). Claims 1, 2, 4-9, 12-14, 23 and 26-35 overcome the rejection under 35 U.S.C. Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. in the same manner as listed above. McBride et al. is cited for disclosing Tc-99m. It is respectfully believed that it is improper to apply an "obviousness to try" standard or indulge in hindsight evaluation or reconstruction to attempt to arrive at the Applicants' claimed Invention. There needs to be a motivation for combining McBride et al. with the other five cited References. Under In re Sang Su Lee, 277 F.3d 1338 (Fed. Cir. 2002), the Federal Circuit found that the Board must not only assure that the requisite findings are made, based on evidence of record, but must also explain the reasoning by which the findings are deemed to support the agency's conclusion. In this case, it is respectfully believed that there is no support to state that a missing claim element, i.e., Tc-99m, is obvious to someone with ordinary skill in the art to simply take this element out of McBride et al. and combine it with five other References to arrive at the Applicant's claimed invention. Moreover, there is no hint or suggestion for combining these References. It is only with hindsight based upon reviewing the Applicant's patent specification that someone would use all of the cited materials in combination. It is respectfully believed that hindsight is not the test of obviousness nor is it alone sufficient that other devices might have been adapted without too much difficulty to produce the object and function of the Applicant's claimed invention.

Therefore, Claims 1, 2, 4-9, 12-14, 23 and 26-35 overcome the rejection under 35 U.S.C.

Section 103(a) over Slaninova et al. in view of Sethi et al. and further in view of the combination of Richards et al., Edmunson et al., and Moroder et al. and further in view of McBride et al. (United States Patent No. 5,620,675).

In addition, the phrase "the group consisting of" has been adding to claim 12 in order to place the language in claim 12 in proper Markush format. No new matter has been added with this amendment.

Therefore, it is now believed that all of the pending Claims, i.e., Claims 1-3, 6-9, 12-14, 23-25, and 27-36, in the present application are in condition for allowance. Favorable action and allowance of the Claims is therefore respectfully requested. If any issue regarding the allow ability of any of the pending Claims in the present application could be readily resolved, or if other action could be taken to further advance this application such as an Examiner's amendment, or if the Examiner should have any questions regarding the present amendment, it is respectfully requested that the Examiner please telephone Applicants' undersigned attorney in this regard.

Respectfully submitted,

Dated: July 26, 2004

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Cholecystokinin receptor imaging using an octapeptide DTPA-CCK analogue in patients with medullary thyroid carcinoma

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Abstract. Cholecystokinin (CCK)-B receptors have been demonstrated on a high percentage of medullary thyroid carcinomas (MTC) in vitro. After encouraging results both in vitro and in animal studies, we studied the efficacy of an octapeptide [^{111}In-DTPA]-CCK analogue in seven patients with MTC. In four of five patients in whom serum calcitonin levels were monitored, a significant rise was found following the injection, indicating retained biological activity of the radiopeptide. In all patients there was visualization of the CCK-B receptor-positive stomach. In one of two patients with known MTC lesions, some of the lesions were visualized; in addition some lesions were visualized in one of the five other patients who had elevated serum tumour markers but negative localizing studies. Radioactivity in the presumed tumour sites was still present at 48 h p.i. The uptake in the presumed tumour sites and stomach was low. Background radioactivity dropped rapidly owing to urinary excretion. After 1 h, breakdown products of the labelled analogue predominated both in urine and in serum, and virtually no intact peptide was present. In conclusion: (1) the CCK-B receptor-positive gastric mucosa and presumed MTC lesions could be visualized in patients using an octapeptide [^{111}In-DTPA]-CCK analogue that is probably internalized, proving the feasibility of CCK-B receptor imaging in vivo; (2) there was a relatively low uptake of the CCK analogue in the strongly CCK receptor-positive stomach, and rapid degradation of the peptide in serum.

Key words: Cholecystokinin – Peptide receptor imaging – CCK receptor – Medullary thyroid carcinoma

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Introduction

Cholecystokinin (CCK) has an important role both in gastrointestinal physiology and in neurotransmission. Its G protein-coupled receptors are divided into CCK-A receptors, which bind CCK, and CCK-B receptors, which bind both CCK and gastrin [1]. CCK-B receptors have also been demonstrated on various tumour cell lines and on primary human tumours, especially of the gastrointestinal tract [2, 3, 4].

Recently, CCK-B receptors were also demonstrated on small cell lung cancers and stromal ovarian cancers as well as on neuronal, renal and myogenic stem cell tumours [5, 6] and human medullary thyroid carcinomas (MTCs) [7]. The presence of CCK receptors on the latter tumour type is probably the molecular basis for the well-established pentagastrin stimulation of serum calcitonin (CT), which provides proof of otherwise undetectable MTC.

Recently, several investigators reported findings of in vitro and animal studies on CCK analogues that may potentially also be used for in vivo human tumour detection. Behr et al. [8, 9] described the visualization of subcutaneous xenografts of a human MTC cell line in nude mice using the radioiodinated heptadecapeptide gastrin-I. Also, in a preliminary study in one patient with MTC, tumour sites were visualized. Reubi et al. [10] tested a number of DTPA- and DOTA-coupled CCK octapeptide analogues and found that two of these had a good affinity in the low nanomolar range for the CCK-B receptor and low affinity (>100 n*M*) for the CCK-A receptor.

Also, both were found to be relatively stable when incubated at room temperature in serum, but not in urine. One of these analogues, code named MP-2288 and linked with either DTPA or DOTA, was also tested extensively in vitro on tumour cell lines and in animal tumour models, with encouraging results [11]. In the present study, we report the results of in vivo scintigraphy with this [^{111}In-DTPA]-CCK analogue in seven patients with biochemical or radiological evidence of MTC.

Materials and methods

Patients. Seven patients with MTC were studied; in six serum CT and/or CEA was elevated, whereas in the seventh a rise in CT levels after pentagastrin testing indicated remnant tumour. Two patients had radiological evidence of metastases before the CCK receptor scintigrams were performed. All patients had undergone thyroidectomy and neck lymph node dissections; three patients had had additional treatments (Table 1).

All patients gave informed consent to participate in the study, which was approved by the hospital's ethical committee.

Methods. The synthesis of DTPA-CCK analogue (DTPA-[D-Asp^{26}, Nle^{28,31}]-CCK(26–33); DTPA-D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2) was described previously [10]. Kits were prepared consisting of 10 μg DTPA-CCK analogue in 10 μl water for injection (WFI), 110 μl 0.2 *M* ammonium acetate and 1 mg gentisic acid in 80 μl 0.05 *M* acetic acid in WFI. Kits were stored at –20°C until use. $^{111}InCl_3$ was added and the mixture was incubated at room temperature for 30 min. The labelling yield was checked using instant thin-layer chromatography (ITLC-SG, Gelman, Ann Arbor, Mich., USA; R_f values for peptide-bound ^{111}In and free ^{111}In-citrate were 0 and 1, respectively) and 0.1 *M* Na citrate, pH 5.0, and the radiochemical purity was checked by high-performance liquid chromatography (HPLC) [μ-Bondapak-C_{18} column (300× 3.9 mm, particle size 10 μm); Waters, Milford, Mass., USA; 5% acetonitrile (ACN), 95% H_2O (0.1% trifluoracetic acid, TFA) 5 min isocratic, thereafter in 30 min to 70% ACN, 30% H_2O (0.1% TFA)]. Labelling yields of ^{111}In-DTPA-CCK analogue ranged between 98.7% and 99.6%, while radiochemical purity (activity bound to the DTPA-CCK analogue, as measured by HPLC) ranged from 91.9% to 96.2%. The remaining activity (3.8%–8.1%) was the sum of unbound radioactivity (about 1%) and ^{111}In bound to a small amount of other peptide fragments that were the result of the peptide synthesis procedure. The mean injected dose was 182 MBq (range 158–211 MBq), coupled to 7.2–8.5 μg DTPA-CCK analogue, except for one patient in whom 18.7 μg was injected.

Imaging. Planar imaging was performed with a dual-headed camera (Picker Int., Cleveland, Ohio, USA) equipped with a medium-energy collimator. The windows were centred over both ^{111}In photon peaks (172 and 245 keV) with a window width of 20%. Dynamic images of the upper abdomen were obtained from the time of injection up to 30 min p.i. Static spot images were obtained 4, 24 and in some patients 48 h p.i. Acquisition time was 15 min.

SPET images of the area(s) of interest were obtained 24 h p.i. using a three-headed camera (Picker Int.). Acquisition consisted of 120 projections, with an acquisition time of 30 s (abdomen) to 45 s (head and thorax) per projection. SPET analysis was performed with a Wiener filter on original data. The filtered data were reconstructed with a Ramp filter. The examination of the scans was done by two of us (E.P.K., D.J.K.) conjointly.

In vivo measurements. The uptakes in the liver, stomach, kidneys and, if applicable, presumed tumour sites, were calculated as described previously [12].

Measurement of radioactivity in blood, urine and faeces. Radioactivity in blood and urine was measured with a COBRA-Packard auto-gamma counting system (Packard, Meriden, Conn., USA).

Blood samples were drawn 10, 20, 40, 60 and 90 min and 2, 4 and 24 h after injection. Urine was collected from 0–1, 1–3, 3–6 and 6–24 h after injection.

The chemical status of the radionuclide in blood and urine was analysed as a function of time by HPLC techniques (see above).

Results

After injection, six patients had a moderate pulse increase of 20–30/min, which lasted for up to 2 min. ECG patterns showed no changes. Two patients had tingling sensations in their extremities and throat, and recognized them as resembling those during pentagastrin testing.

In all patients, visualization of heart, kidneys and liver was seen in the first 30 min p.i., and gradually the stomach was also visualized. At 4 h p.i. soft tissue accumulation, especially in the breasts and nipples, was seen, and there was clearer visualization of the stomach

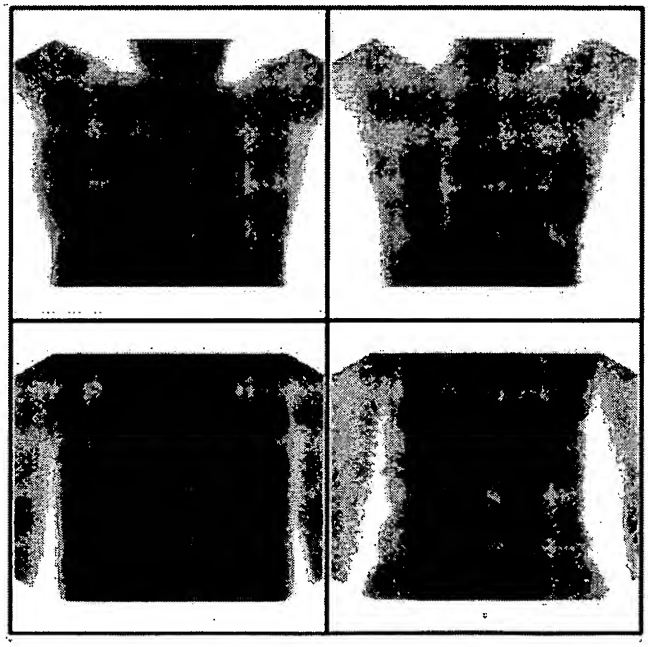

Fig. 1. CCK receptor imaging 4 h p.i. in two patients. Each column represents one patient. Anterior chest images (*upper panel*) show relatively high soft tissue accumulation. Note the uptake in the breasts of the female patient (*right side*). The stomach is visualized in the *lower panel*, with variable uptake in the liver and activity in the bowel. The patients were not laxated

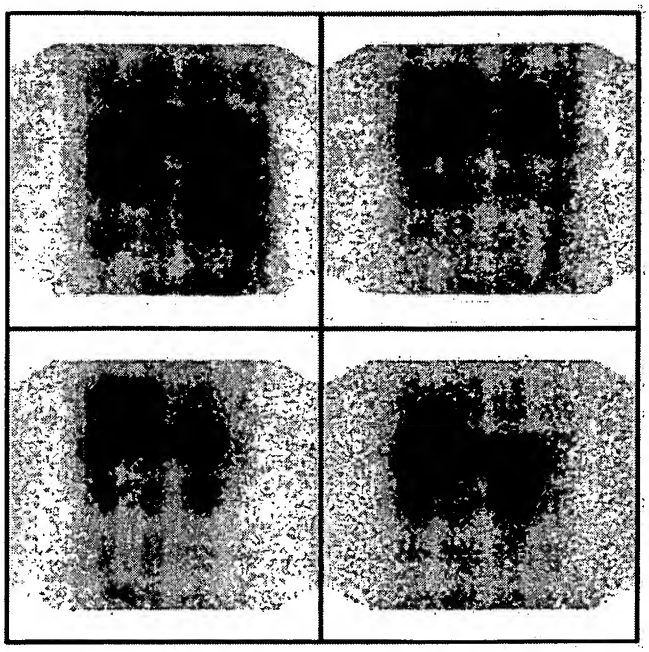

Fig. 2. Anterior abdominal images in two patients at 24 h p.i. (*upper panel*) and 48 h p.i. (*lower panel*). There is visualization of the stomach and liver, and variable bowel radioactivity, at both time points. The count density is low

(Fig. 1). Stomach visualization was still present at 24 h and, in those patients in whom images were acquired at that time, at 48 h p.i. (Fig. 2). Presumed pathology-related uptake in two patients was clearly recognized after 24 h when background radioactivity had dropped considerably, and was still present at 48 h p.i. (Fig. 3, Table 1). Known lesions in liver and bone were not recognized in patient 1. A biopsy of one of the liver metastases was also CCK-B receptor-negative during in vitro receptor autoradiography (data not shown). Liver metastases in patient 2 showed low or absent accumulation of the radiopharmaceutical, as demonstrated with dual-isotope ^{99m}Tc-Albures and [^{111}In-DTPA]-CCK analogue SPET scanning at 48 h. Presumed pathology in patient 5, detected with CCK receptor scintigraphy, was correlated with a retrosternal mass measuring 8×4 mm on thin-slice computed tomography scanning performed because of the findings during CCK scintigraphy.

Mean count density in the images was low, ranging from 68 to 312 kcounts/15 min at 24 h, depending on the part of the body that was imaged (Table 2). For comparison, these values are about fourfold higher after injection of 200 MBq [^{111}In-DTPA-D-Phe^1]-octreotide (Table 2). The uptake in stomach, kidneys, liver and presumed pathology, expressed as percentage of injected dose, is listed in Table 3. Because of the low count rate in most organs, these figures should be interpreted with caution. In comparison with somatostatin receptor scintigraphy using [^{111}In-DTPA-D-Phe^1]-octreotide (SRS), the uptake in liver and kidneys was much lower during CCK receptor imaging.

The response of serum CT concentrations following the injection of the radiolabelled CCK analogue was monitored in five patients. In four of them a significant rise in CT levels was found; however, in only one of these patients was presumed pathology visualized during scintigraphy. In patient 1, with known extensive tumour load that was not visualized during scintigraphy, CT levels remained unchanged.

Serum radioactivity was measured in five patients (Fig. 4). In comparison with radioactivity levels mea-

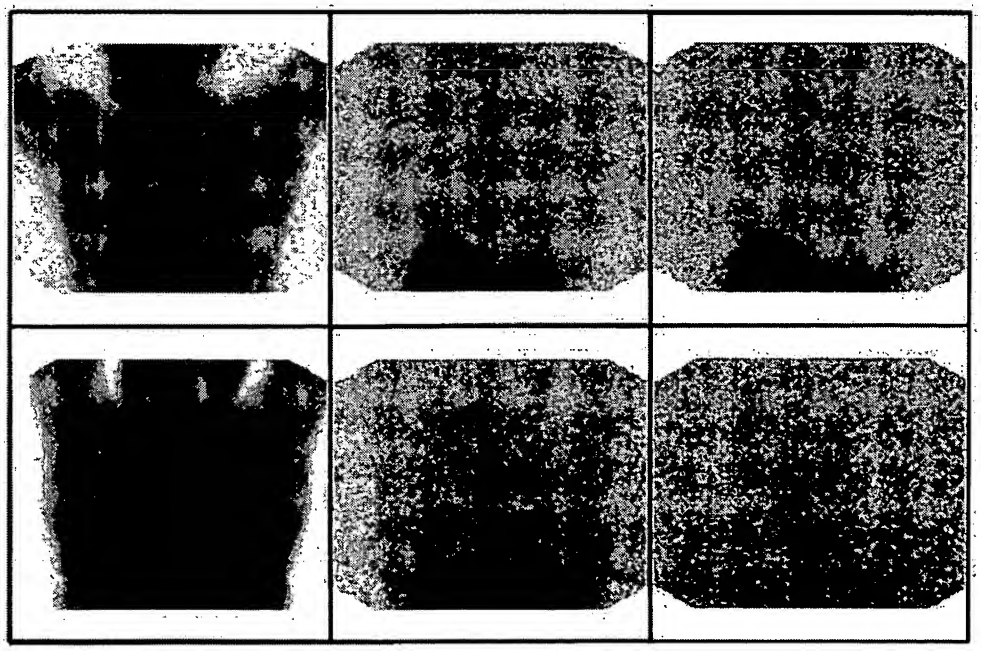

Fig. 3. Uptake in presumed pathology in two patients (patients 5 and 2, Table 1). There is hardly any or no visualization of the lesions at 4 h p.i. (*left column*), and clear uptake at 24 h (*middle column*) and 48 h p.i. (*right column*). Note the low count density at later time points.

Table 1. Patient data, hormone levels and imaging results

Patient	Duration of MTC (years)	Prior treatment[a]	CT (μg/l)[b]	CEA (μg/l)[b]	Known lesions	Lesions on CCK receptor imaging
1	6	Surgery, ^{131}I, RT, ^{111}In-octreotide treatment	767.0	35.8	Bone, liver, bone marrow	None
2	1	Surgery	19.0	52.0	Neck, liver	Chest, liver (some)
3	18	Surgery, hemihepatectomy	4.5	15.3	None	None
4	5	Surgery	1.1	4.4	None	None
5	8	Surgery	6.2	8.6	None	Chest
6	24	Surgery	2.3	39.5	None	None
7	1	Surgery, ^{131}I	0.05	0.6	None	None

[a] Surgery includes thyroidectomy and neck lymph node dissections. ^{111}In-octreotide treatment: treatment with high doses (6–7 GBq per treatment) [^{111}In-DTPA-D-Phe^1]-octreotide. RT, radiation therapy

[b] Normal values: CT <0.14 μg/l; CEA <10 μg/l

Table 2. Mean (SEM) kcounts/view (15 min) after [^{111}In-DTPA]-CCK analogue, compared with those during SRS using [^{111}In-DTPA-D-Phe^1]-octreotide

	CCK receptor imaging				SRS	
	No.	4 h	24 h	48 h	No.	24 h
Head	7	467 (85)	68 (2)	61 (3)	5	262 (48)
Chest	7	1187 (228)	190 (23)	127 (12)	5	956 (202)
Abdomen	7	1631 (323)	312 (36)	209 (25)	5	1303 (108)

The 48 h column of CCK receptor imaging pertains to fewer patients. The SRS studies contained no pathology with intense uptake

Table 3. Mean (SEM) uptake, expressed as percentage of the injected dose, in organs and presumed pathology during CCK receptor imaging, compared with data from Krenning et al. [13] during SRS using [^{111}In-DTPA-D-Phe^1]-octreotide

	CCK receptor imaging				SRS		
	n	4 h	24 h	48 h	4 h	24 h	48 h
Liver	7	0.43 (0.10)	0.73 (0.15)	0.88 (0.21)	1.9	3.0	2.5
Stomach	6	0.39 (0.10)	0.32 (0.07)	0.25 (0.04)	–	–	–
Kidneys	5	0.88 (0.33)	0.28 (0.03)	0.24 (0.03)	7.2	4.8	3.5
Pathology	2	0.019/0.000	0.005/0.001	0.005/0.001			

The 48 h column of CCK receptor imaging pertains to four patients. Percentages in SRS columns are means derived from 6 or 18 patients

sured after the injection of [^{111}In-DTPA-D-Phe^1]-octreotide, derived from a previous study [14], these levels dropped more rapidly and profoundly after [^{111}In-DTPA]-CCK analogue injection. Also, urinary excretion of radioactivity was more rapid in the first 6 h after injection than after [^{111}In-DTPA-D-Phe^1]-octreotide, although it was comparable after 24 h, reaching more than 90% (Fig. 5).

HPLC analysis of urine in two patients showed 8.3% of the radioactivity at the CCK peptide peak in urine collected up to 1 h p.i., and <1% in urine collected from 1 to 3 h p.i. (data not shown). HPLC analysis of serum, taken at 1 h p.i. in two patients, demonstrated 1.5% and <1% of radioactivity at the CCK peptide analogue peak (data not shown).

Discussion

New radiopharmaceuticals are needed to warrant more widespread use of peptide receptor scintigraphy. To date, the only commercially available kit for such scintigraphy is OctreoScan, valuable if applied in patients with somatostatin receptor-positive tumours, but applicable in only a minority of cancer patients.

All the results of the preclinical studies with the chelated CCK analogue used in the present study seemed encouraging: In vitro studies showed a high affinity for the CCK-B receptor, the peptide proved stable when kept in human serum at room temperature, internalization in a CCK-B receptor-positive cell line was demonstrated, and rapid renal clearance and relatively high tumour to blood

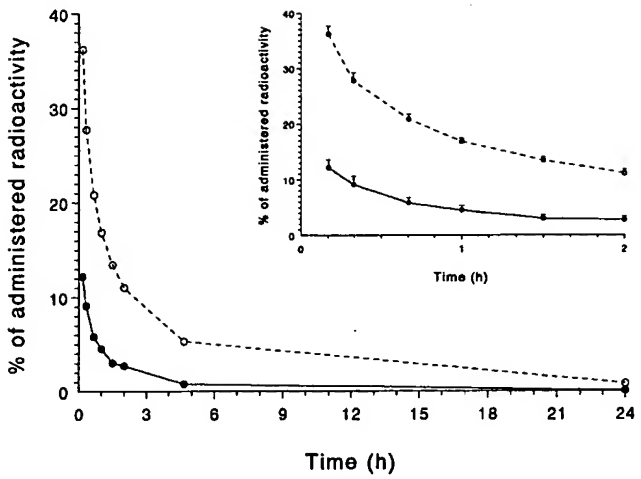

Fig. 4. Mean serum radioactivity, expressed as percentage of the injected dose, after [^{111}In-DTPA]-CCK analogue in five patients (*closed dots, solid line*), compared with that after [^{111}In-DTPA-D-Phe^1]-octreotide in four other patients (*open dots, stippled line*) from a previous study [14]. The *inset* shows means and SEMs

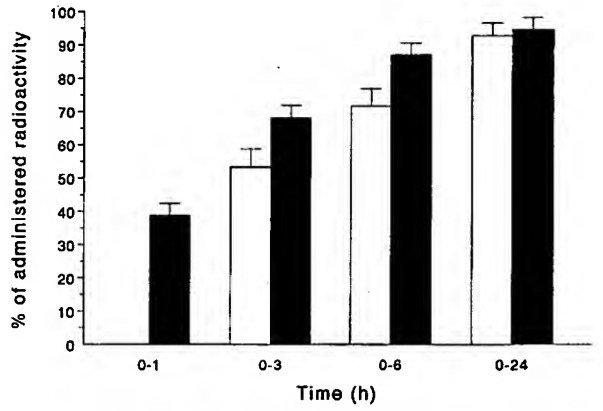

Fig. 5. Mean (SEM) cumulative radioactivity in urine after [^{111}In-DTPA]-CCK analogue in seven patients (*closed bars*) compared with that after [^{111}In-DTPA-D-Phe^1]-octreotide in six other patients (*open bars*) from a previous study [14]

ratios were shown in tumour-bearing animals [10, 11]. Yet the expectations raised by these experiments were not fully met when the peptide was clinically tested. As in the preclinical studies, rapid binding to CCK-B receptor-positive gastric mucosa was observed in the present patient study. Also, as in animal studies, rapid renal clearance was found. Breakdown products of the peptide were found in the urine, in accordance with the preclinical data reported by Reubi et al. [10]. However, the HPLC profile in serum collected 1 h p.i. indicated less than 2% intact peptide, in contrast to the reported stability of the peptide in serum in vitro [10]. This rapid degradation of the peptide in vivo may be due to breakdown in the liver, as this organ was visualized in all patients. Such organ-mediated degradation would also explain the difference between in vivo and in vitro findings.

Despite the rapid degradation of the CCK analogue in vivo, the CCK-B receptor-positive stomach was visualized in all patients, as well as presumed MTC tumour sites in two of them. Moreover, a rise in serum CT after injection of the peptide was found in four of five patients. These data point to the following conclusions: first, that the administered analogue is biologically active, and second, that, in accordance with preclinical studies [11], internalization of the peptide takes place (because the visualization of CCK-B receptor-positive tissues is present up to 48 h p.i., whereas degradation of the peptide in serum is observed within 1 h p.i., the receptor-bound peptide must obviously be protected against degradation by means of internalization).

Two of our patients had known tumour localizations, yet in only one of these was visualization of some of the known lesions observed during CCK receptor scintigraphy. Also, scintigraphy suggested possible pathology in only one of the five remaining patients. However, it should be stressed that in those patients no MTC sites were detected with standard imaging methods, though their MTC was evidenced by elevated serum markers or abnormal pentagastrin tests. These somewhat disappointing results contrast with the high incidence of CCK-B receptors in 21 of 23 MTCs in vitro (92%) reported by Reubi and Waser [7] and CCK-B mRNA in six of six MTCs reported by Amiri-Mosavi et al. [15]. In these studies, however, the majority of MTCs that were studied were primary tumours in the thyroid. This implies that this in vitro study was performed in mostly well-differentiated MTCs. It is well known that somatostatin receptors are present on well-differentiated MTC, but not on dedifferentiated forms [16, 17]. No in vitro data are available for the CCK-B receptor status in dedifferentiated MTC or MTC metastases. Our in vivo results could therefore be due not only to the application of a less ideal radiolabelled peptide, but also to a lower percentage of CCK-B receptor-positive MTCs and/or a lower CCK-B receptor density in these tumours when dedifferentiated MTCs are also taken into account.

In patients with MTC, the primary goal of peptide receptor scintigraphy is to demonstrate small lesions that are not detected with conventional imaging techniques. This is the reason why we selected patients with small lesions that had not been localized with other imaging techniques. The secondary goal may be a high accumulation of the radiopharmaceutical in the tumour in order to perform radiotherapy with labelled peptides. Both goals, which were perhaps set too high when testing a new radiopharmaceutical, were missed with the CCK analogue that we used. Behr et al. initially described an in vivo patient study using ^{131}I-gastrin-I [8]. Though tumour uptake was found, the tumour to background ratio was low, to judge from the published figure. In a recent report, the same group presented preliminary data using ^{111}In-labelled chelated minigastrin in three patients [9]. Like us, they found uptake especially in the stomach and kid-

neys, and to a lesser extent in the liver. The uptake of the peptide analogue in the CCK receptor-positive stomach is encouraging, as it proves the feasibility of CCK receptor imaging. The scintigraphic visualization of the stomach indicates that the CCK-B receptor can be detected in vivo in man. This is not a good indicator of the sensitivity of the method for detecting MTC, since the CCK-B receptor density in the stomach is very high [18], i.e. higher than in most MTCs. Nevertheless, to judge from the published scan example and also the findings of our study, the uptake in the tumour seems low, as does the tumour to background ratio. Therefore, other chelated CCK analogues that are more stable in vivo and are internalized are needed.

From this study we draw the following conclusions: (1) The CCK-B receptor-positive gastric mucosa and presumed MTC lesions could be visualized in patients using an 8-amino acid [^{111}In-DTPA]-CCK analogue that is probably internalized, proving the feasibility of CCK-B receptor imaging in vivo. (2) There was a relatively low uptake of the CCK analogue in the strongly CCK receptor-positive stomach, and a rapid degradation of the peptide in serum.

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Unsulfated DTPA- and DOTA-CCK analogs as specific high-affinity ligands for CCK-B receptor-expressing human and rat tissues in vitro and in vivo

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Abstract. Receptors for regulatory peptides such as somatostatin or vasoactive intestinal polypeptide are expressed by a number of human neoplasms and can be visualized in vivo with peptide receptor scintigraphy. Recently, the CCK-B receptor, which binds both gastrin and cholecystokinin with high affinity, was shown using in vitro methods to be overexpressed in a number of human tumor tissues, including medullary thyroid carcinomas, small cell lung cancers, astrocytomas, gastrointestinal tumors, and stromal ovarian cancers. In the present study, we have designed novel, unsulfated CCK octapeptide analogs linked to the metal chelating DTPA and DOTA, and have tested them for their binding affinity to CCK-B receptor-positive tissue from human tumors: The most potent compounds assayed were DTPA-[Nle^{28,31}]-CCK(26–33) (MP2286) and DTPA-[D-Asp^{26},Nle^{28,31}]-CCK(26–33) (MP2288) with an IC_{50} of 1.5 n*M*. For comparison, analogs with C-terminal DTPA, such as [Nle^{28,31},Aphe^{33}(*p*-NH-DTPA)]-CCK(26–33) and CCK-(26–33)-NH$(CH_2)_2$ NH-DTPA, had an IC_{50} of >100 n*M*. DOTA-[D-Asp^{26},Nle^{28,31}]-CCK(26–33) had an IC_{50} of 3.9 n*M*. The compounds were selective for CCK-B receptors as they did not bind with high affinity to CCK-A receptors expressed in human tumors (meningiomas or gastroenteropancreatic tumors). In vivo rat biodistribution studies with indium-111 labeled MP2286 and MP2288 showed that the primary mode of clearance was renal, and the primary sites of uptake (% ID/g 24 h p.i.) were kidneys (0.270 and 0.262, respectively) and the gastrointestinal tract. The CCK-B receptor-expressing gastric mucosa showed specific in vivo accumulation of ^{111}In-labeled MP2288 which could be blocked in the presence of excess unlabeled MP2288. ^{111}In-labeled MP2286 and MP2288 were also found to be stable in human plasma whereas both compounds were degraded in urine (>40% after 3 h at 37°C). The affinity, specificity, biodistribution, and stability of these two DTPA-CCK analogs indicate that these compounds have substantial promise for use in the in vivo visualization of CCK-B receptor-expressing tumors.

Key words: Cholecystokinin-B receptors – ^Metal chelating ligands – Cholecystokinin – Octapeptide – Biodistribution – Tumor targeting

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Introduction

The successful application of radiolabeled somatostatin and vasoactive intestinal peptide analogs for the in vivo imaging of receptor-expressing tumors [1, 2] has stimulated the search for other disease-associated peptide receptors [3, 4]. Peptides which can be used to target abundant peptide receptors, particularly those which are selectively expressed, may be especially useful as tools in nuclear medicine for the development of new diagnostic imaging agents and/or radiotherapeutics. Recently, we have evaluated cholecystokinin (CCK)-A and CCK-B receptors in the normal human gastrointestinal tract [5] as well as in a variety of human tumors [6, 7]. It was observed that specific tumor types frequently express CCK-B receptors, namely medullary thyroid carcinomas (MTCs), small-cell lung cancers (SCLCs), astrocytomas, stromal ovarian tumors, and some gastroenteropancreatic tumors [7]. We were able to design an iodinated CCK analog, the nonsulfated ^{125}I[D-Tyr-Gly, Nle^{28,31}]-CCK(26–33), which retained high affinity in the nanomolar range and showed high specificity for human CCK-B receptors [7], as a potential radioligand for in vivo scintigraphy. However, for routine use in diagnostic nuclear medicine, it would be preferable to have CCK analogs linked to a chelator, such as DTPA or DOTA, which can then be labeled with indium-111 or other clinically useful radioisotopes. This approach would represent further progress, as observed previously with Octreoscan [1].

In the present study, we therefore designed and synthesized a number of CCK derivatives linked to DTPA or DOTA. These compounds were tested for their affinity for CCK-A and -B receptors expressed by human tumors, using in vitro receptor autoradiography. The compounds with the highest binding affinities were further tested in vitro for their stability in human plasma and urine and in vivo for their biodistribution behavior in normal rats.

Materials and methods

General method for the solid phase synthesis of CCK analogs

Solid phase peptide synthesis was performed using an Applied Biosystems Model 432 A Synergy Peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) strategy [8]. Instrument protocol required resin containing 25 mmol of Fmoc-amine content and 75 mmol of subsequent Fmoc-protected amino acids activated by a combination of *N*-hydroxylbenzotriazole (HOBt)/(2-(1-H benzotriazol-1-yl)-1,1,1,3-tetramethyluronium hexafluorophosphate (HBTU). Three letter codes for common amino acids are used. All the standard Fmoc-protected amino acids were purchased commercially unless otherwise stated. The unusual amino acids used have the following abbreviations: *p*-aminophenylalanine, Aphe; 2,3-diaminopropionic acid, β-Dpr. Fmoc-(*p*-Alloc)-Aphe-OH was prepared from Fmoc-Aphe-OH and allyloxycarbonyl chloride by standard procedures.

Rink amide resin was used for the synthesis of C-terminal carboxamide formation unless otherwise stated. After the synthesis was completed, the peptides were cleaved from the resin and deprotected using a mixture comprising trifluoroacetic acid:phenol:thioanisole:water (85:5:5:5) for 6–10 h at room temperature. The products were precipitated by *t*-butyl methyl ether and centrifuged. The peptide-resin mixture was washed with *t*-butyl methyl ether and centrifuged 5–6 times to remove residual cleavage mixture. Acetonitrile:water (2:3) was added to the residue and filtered to remove the resin. The filtrate containing the crude peptide was lyophilized and pure peptides were obtained by preparative liquid chromatography. Molecular weight determination was done by mass spectrometry operating in electrospray mode (ESI).

Method A. For the incorporation of DTPA (diethylene triamine penta-acetic acid), the N-terminal Fmoc-protecting group was removed in the synthesizer. Tri-*t*-butyl-DTPA (75 mmol) was placed at the appropriate location in the synthesizer and activated similar to other amino acids for coupling [9]. Cleavage, deprotection, and isolation of the peptides were carried out as outlined above.

Method B. Incorporation of *p*-(DTPA-NH)-Phe at the C-terminus: The peptide synthesis was carried out as described above with the incorporation of Fmoc-(*p*-Alloc)-Aphe-OH at position 8 and *t*-Boc-Asp(OtBu)-OH at the N-terminus. The resin containing the protected octapeptide was treated with $Pd(PPh_3)_4$ in chloroform:acetic acid:N-methyl morpholine (37:2:1) to remove the allyloxycarbonyl protecting group [10]. The resin was transferred back to the synthesizer and condensed with tri-*t*-butyl-DTPA as described in method A. At the end of the synthesis, MP-2336 was isolated according to the general method described above.

Method C. Incorporation of DTPA at the C-terminus: The protected peptide containing *t*-Boc-Asp(OtBu)-OH at the N-terminus was prepared according to the standard protocol above using diaminoethane-trityl resin. At the end of the synthesis, the protected peptide was obtained from the resin using 3×2 ml of 1% trifluoroacetic acid in methylene chloride. Trifluoroacetic acid was neutralized with 5 ml of 5% triethylamine, evaporated to dryness, and dissolved in 1 ml of *N,N*-dimethylformamide (DMF). To this solution, 75 mmol of tri-*t*-butyl-DTPA anhydride in DMF (prepared from 75 mmol of tri-*t*-butyl-DTPA and 75 mmol of dicyclohexylcarbodiimide in 1 ml of DMF) was added, and the solution was shaken for 2–3 h. DMF was removed under reduced pressure. Deprotection and isolation of MP-2312 was carried out as described in general methods.

Method D. Incorporation of DOTA at the N-terminus: An identical procedure to that described in method A was followed for the incorporation of DOTA at the N-terminus. Tri-*t*-butyl DOTA was placed in the synthesizer instead of tri-*t*-butyl-DTPA, and the activation was carried out in a similar manner. Tri-*t*-butyl DOTA was synthesized internally by a modification of the procedure of Mishra et al. [11]. At the end of the synthesis, MP-2354 was isolated according to the general method described above.

The structures of the synthesized CCK analogs are shown in Table 1. The various structural modifications were chosen in order (1) to improve the peptide stability by modifying or replacing those amino acids most susceptible to facilitate peptide degradation, (2) to identify an adequate location to attach a chelator molecule without loss of receptor affinity.

Evaluation of binding affinities with in vitro receptor autoradiography

Human tumors known from previous studies [7] to express either CCK-A or CCK-B receptors were used. All tissues were frozen immediately after surgical resection and stored at –70°C. Receptor autoradiography was performed on 10- and 20-μm thick cryostat (Leitz 1720, Rockleigh, N.J.) sections of the tissue samples, mounted on microscope slides, and then stored at –20°C for at least 3 days to improve adhesion of the tissue to the slide, as described elsewhere [7]. Each tissue underwent receptor autoradiographic processing with ^{125}I-D-Tyr-Gly-Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-amide (^{125}I-CCK), a radioligand identifying both CCK-A and -B receptors, as described previously [5, 7]. The sections were preincubated in 50 mmol/l Tris-HCl, 130 mmol/l NaCl, 4.7 mmol/l KCl, 5 mmol/l MgCl, 1 mmol/l ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*,-tetra-acetic acid, and 0.5% bovine serum albumin (BSA), pH 7.4 (preincubation solution), for 30 min at 25°C. The slides were then incubated in a solution containing the same medium as the preincubation solution minus the BSA, and the following compounds were added: 0.025% bacitracin, 1 mmol/l dithiothreitol, 2 μg/ml chymostatin, 4 μg/ml leupeptin, pH 6.5, and the radioligand, 45 pmol/l ^{125}I-CCK (2000 Ci/mmol; Anawa). The slides were incubated at room temperature with the radioligand for 150 min. Increasing amounts of nonradioactive, sulfated CCK-8, gastrin, or each of the newly designed DTPA- or DOTA-CCK analogs were added to the incubation medium to generate competitive inhibition curves. On completion of the incubation, the slides were washed 6 times for 15 min each in ice-cold preincubation solution, pH 7.4. The slides were rinsed twice in ice-cold distilled water for 5 s each. They were then dried under a stream of cold air at 4°C, apposed to ^{3}H-hyperfilms, and exposed for 1–7 days in X-ray cassettes.

The same protocol was used for the CCK-A and CCK-B receptor analysis of the rat stomach, jejunum, ileum, and colon [5].

The autoradiographs were quantified using a computer-assisted image-processing system, as described elsewhere [12]. Tissue standards for iodinated compounds (Amersham, Little Chalfont, UK) were used for this purpose.

The selection of tumors expressing the adequate CCK receptor was performed as reported previously [7]. Tumors were considered as expressing CCK-A receptors when the ^{125}I-CCK analog was fully displaced by 50 n*M* sulfated CCK-8 but not displaced by 50 n*M* gastrin. Conversely, tumors were considered as expressing CCK-B receptors when the ^{125}I-CCK ligand was fully displaced by nanomolar concentrations of sulfated CCK-8 and gastrin. Tumors expressing concomitantly CCK-A and -B receptors were not used in this study.

The two MP2286 and MP2288 analogs used in displacement studies were labeled with ^{115}In as follows: The ^{115}In-peptide complex was prepared by reacting MP2288 or MP2286 (70 nmol) with $^{115}InCl_3$ (105 nmol) in 200 µl of 0.005 *N* HCl for 30 min at room temperature. The solution was lyophilized to dryness and redissolved in 5 m*M* $NaHCO_3$ and analyzed by reverse phase high-performance liquid chromatography (HPLC) and mass spectrometry. The analysis indicated that the peptide was >99% ^{115}In-complex.

In vivo biodistribution in rats

The overall biodistribution properties of ^{111}In-labeled MP2286 and MP2288 were determined in rats. Peptide radiolabeling was performed in 25 m*M* NaOAc, 12.5 m*M* Na-ascorbate, pH 5.0. Typically, reactions were carried out using 1 mCi $^{111}InCl_3$ and 1 µg peptide in a total volume of 25 µl. After incubation for 15 min at room temperature the radiolabeled peptides were diluted to desired volumes for injection using sterile phosphate buffered saline (PBS) containing 5% ethanol. Radiolabeling efficiency was greater than 99%, as measured with reverse phase HPLC on a Novo-Pak C_{18} column, 3.9×150 mm (Waters), using a 15-min linear gradient of 0 to 70% solvent B (solvent A, 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B, 90% acetonitrile, 0.1% trifluoroacetic acid). For each compound, nine normal female Sprague-Dawley rats (180–200 g) in groups of three were anesthetized with Metofane gas and injected via the jugular vein with 25 µCi of ^{111}In-labeled peptide. At 1, 4, and 24 h post injection, groups of rats were sacrificed and, after collection of a blood sample by cardiac puncture, tissue samples were removed for radioassay. The % injected dose per gram tissue was calculated against standard dilutions of the radiolabeled compounds.

In vivo blocking experiments

Animals were injected with ^{111}In-MP2288 prepared at high and low radio-specific activity to determine whether uptake into CCK-B receptor-expressing target tissues is specific. High specific activity (HSA), 1400 Ci/mmol ^{111}In-MP2288 was prepared as indicated above. The same radiolabeled material was used to prepare low specific activity peptide (~0.5 Ci/mmol) by addition of 0.5 mg of unlabeled peptide to 180 µCi of radiolabeled material. Two sets of three rats (fasted for 24 h) were anesthetized and then injected with 50 µCi of high or low radio-specific activity peptide. The animals were imaged by gamma scintigraphy at various times post injection. At 120 min post injection, the animals were sacrificed, and the gastrointestinal tract from the stomach to the distal large intestine was removed, rinsed with distilled water, positioned on paper, photographed, and then scintigraphed. Stomach tissue has been reported to express the highest density of CCK-B receptors [5] apart from the brain; therefore, after the above scintigraphic imaging, individual stomachs with contents were weighed and total radioactivity measured in a gamma counter. The stomachs were then cleaned and rinsed with distilled water and then reweighed and counted to determine the radioactivity associated specifically with tissue.

In vitro stability of CCK analogs in human urine and plasma

The stability of the analogs in urine was determined using 10× concentrated human urine prepared using a 10,000-dalton cutoff, YM10 Amicon membrane. Reactions contained 10 µl concentrated urine and radiolabeled peptide (see above), in a total volume of 100 µl of PBS, pH 7.2. Peptides were incubated for 3 h at 37°C and then analyzed by reverse phase HPLC on a Novo-Pak C_{18} column, 3.9×150 mm (Waters), using a 15-min linear gradient of 0 to 70% solvent B (solvent A, 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B, 90% acetonitrile, 0.1% trifluoroacetic acid). Similar stability tests were performed using human plasma (Calbiochem). Three-hour incubations were as described above except that plasma was used in each 100 µl reaction.

Results

The binding affinities of nine nonsulfated, DTPA-linked CCK analogs and one DOTA-CCK analog were determined by measuring the displacement of receptor-bound ^{125}I-labeled CCK in tumor tissue sections expressing CCK-A and CCK-B receptors. The measured IC_{50} values are listed in Table 1. All of the tested compounds with an N-terminal DTPA (MP2247, MP2286, MP2288, MP2290, MP2296, MP2294) showed very high affinity binding to CCK-B receptors. The compound MP2354, an N-terminal-DOTA-CCK analog, also displayed very high affinity for CCK-B receptors. The compound MP2292, with L-Trp replaced with D-Trp, had a marked reduction in observed affinity. Also, CCK analogs with DTPA conjugated at or near the C-terminus (MP2336 and MP 2312, respectively) had very low binding affinity for the CCK-B receptor (Table 1). None of the synthesized compounds showed any significant affinity for the CCK-A receptor (Table 1). Figure 1 shows a displacement experiment with the various CCK analogs using a CCK-B receptor-expressing MTC and a CCK-A receptor-expressing meningioma, relative to sulfated CCK-8 and gastrin as control substances. Their rank order of displacement potencies corresponds closely to the values listed in Table 1. MP2288 and/or MP2286, which differ only by the presence of L-Asp (MP2286) or D-Asp at the N-terminus (MP2288), have very high binding affinities in all the tested tumor types expressing CCK-B receptors (MTCs, SCLCs, astrocytomas, gastroenteropancreatic tumors, stromal ovarian tumors). Furthermore, chelation of the DTPA ligand with ^{115}In in MP2288 or MP2286 does not affect the binding affinity for the CCK-B recep-

Table 1. Structures and binding properties (IC_{50}) of various DTPA- and DOTA-CCK analogs to human tumoral CCK-A and CCK-B receptors (substituted aminoacids are in italic)

Code Nr.	Synthesis method	Structures	Binding properties	
			CCK-B-R IC_{50} (n*M*)	CCK-A-R IC_{50} (n*M*)
MP2247	A	DTPA-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	2.3	>100
MP2286	A	DTPA-Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	1.5	>100
MP2288	A	DTPA-*D*Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	1.5	>100
MP2290	A	DTPA-*D*Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	2.8	>100
MP2296	A	DTPA-Asp-Tyr-Thr-Gly-Trp-*Nle*-Asp-Phe-NH_2	4.5	>100
MP2294	A	*Dpr*-(β-DTPA)-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	3.5	>100
MP2292	A	DTPA-*D*Asp-Tyr-Nle-Gly-*D*Trp-Nle-Asp-Phe-NH_2	42.0	>100
MP2312	B	Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-*p*(DTPA-NH)Phe-NH_2	>100	>100
MP2336	C	Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH-$(CH_2)_2$-NH-DTPA	>100	>100
MP2354	D	DOTA-*D*Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	3.9	>100

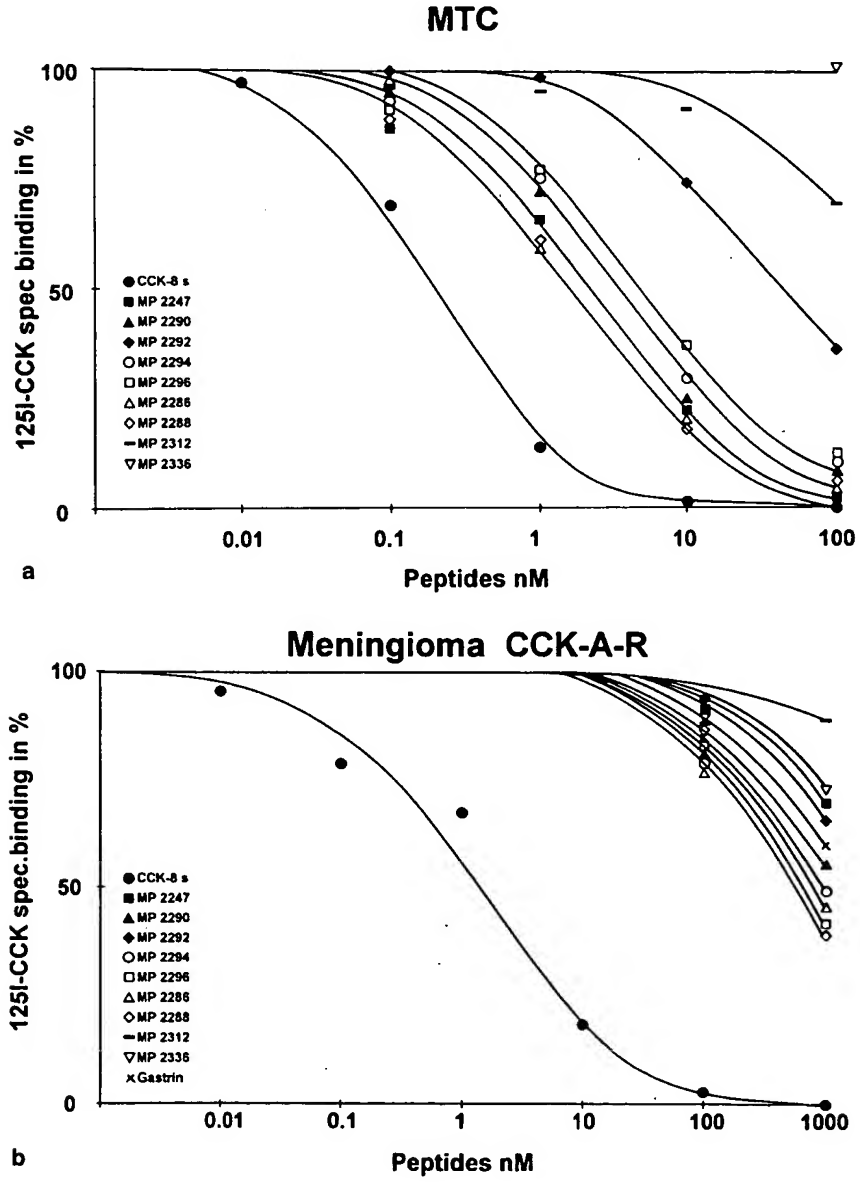

Fig. 1A, B. Competition experiments using ^{125}I-CCK as radioligand with increasing concentrations of sulfated CCK-8 or gastrin as controls, and increasing concentrations of the nine different DTPA-CCK analogs (listed in Table 1). **A** Tissue sections from a CCK-B receptor-expressing MTC; **B** tissue sections from a CCK-A receptor-expressing meningioma. Each tissue section was incubated with 45 pmol/l ^{125}I-CCK. Each *point* represents the radioligand binding to tumor tissue as determined by absorbance in autoradiographs. Nonspecific binding was subtracted from all values

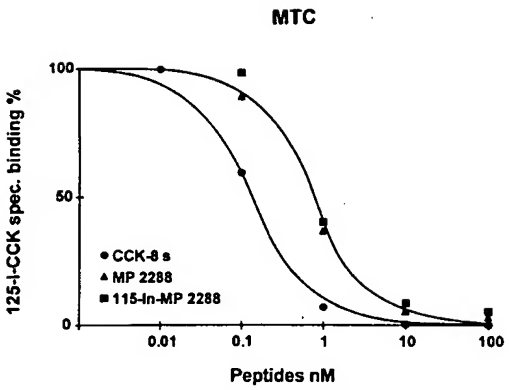

Fig. 2. Competition experiment with sulfated CCK-8 (control), MP2288, and ^{115}In-MP2288 in a CCK-B receptor-expressing MTC. Same experimental conditions as described in Fig. 1

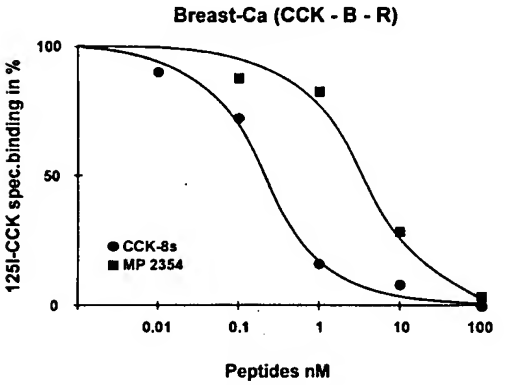

Fig. 3. Competition experiment with sulfated CCK-8 and the DOTA analog MP2354 in a CCK-B receptor-expressing breast cancer. Same conditions as in Fig. 1

tor, as seen in a CCK-B receptor-expressing MTC (Fig. 2). Substitution of the DTPA chelator with DOTA also had no major effect on binding affinity, as demonstrated by the IC_{50} value determined for MP2354 using a breast tumor sample expressing CCK-B receptors (Table 1, Fig. 3).

MP2286 and MP2288 were investigated further for their biodistribution properties in normal rats and for their stability in human urine and blood plasma. Tables 2 and 3 and Fig. 4 show the biodistribution of these two compounds as the mean percentages of the injected dose (% ID) per gram tissue. For both compounds tested, the blood and peripheral soft tissues such as kidneys, muscle, spleen, heart, and small intestine showed very similar localization and low retention patterns. Blood clearance was rapid for both compounds over the 24-h time course studied. The bone (femur) tissue also showed a very similar pattern of localization and very low level of retention for both compounds. MP2286 cleared slightly more rapidly from the liver, pancreas, and the lungs, compared with MP2288. The stomach and contents indicated a higher value at 60 min post injection for

Table 2. Percent injected dose of ^{111}In-MP2288 per gram of tissue sample (% ID/g±SE; n=3)

Tissue	Time post injection		
	1 h	4 h	24 h
Blood	0.062±0.008	0.014±0.001	0.004±0.000
Liver	0.086±0.009	0.099±0.013	0.042±0.002
Kidneys	0.402±0.035	0.261±0.016	0.262±0.009
Skeletal muscle	0.013±0.001	0.004±0.000	0.004±0.000
Bone	0.028±0.003	0.013±0.001	0.014±0.001
Spleen	0.027±0.001	0.019±0.002	0.021±0.001
Heart	0.022±0.003	0.006±0.000	0.005±0.000
Lung	0.053±0.010	0.025±0.007	0.019±0.008
Pancreas	0.050±0.003	0.047±0.010	0.027±0.003
Stomach[a]	0.303±0.106	0.079±0.024	0.099±0.030
Small intestines[a]	0.101±0.029	0.026±0.007	0.019±0.005
Large intestines[a]	0.141±0.086	0.123±0.035	0.151±0.065

[a] Includes contents

Table 3. Percent injected dose of ^{111}In-MP2286 per gram of tissue sample (% ID/g±SE; n=3)

Tissue	Time post injection		
	1 h	4 h	24 h
Blood	0.066±0.008	0.010±0.000	0.005±0.000
Liver	0.043±0.004	0.029±0.001	0.023±0.001
Kidneys	0.428±0.023	0.331±0.008	0.270±0.015
Skeletal muscle	0.014±0.001	0.004±0.000	0.004±0.000
Bone	0.032±0.004	0.012±0.001	0.011±0.000
Spleen	0.030±0.002	0.022±0.001	0.022±0.001
Heart	0.023±0.002	0.006±0.000	0.004±0.000
Lung	0.050±0.002	0.014±0.001	0.008±0.000
Pancreas	0.039±0.002	0.023±0.001	0.017±0.002
Stomach[a]	0.083±0.004	0.123±0.027	0.063±0.007
Small intestines[a]	0.108±0.030	0.038±0.007	0.014±0.002
Large intestines[a]	1.231±0.353	0.412±0.146	0.166±0.040

[a] Includes contents

MP2288, but at 4 and 24 h post injection the two compounds distributed in a similar pattern. The uptake into the large intestines showed a greater discrepancy between the two compounds, with MP2286 showing almost ninefold greater accumulation than MP2288 at 1 h post injection. However, at 24 h post injection the distributions into this organ were nearly identical (Tables 2, 3). The uptake into the large intestines was determined without removal of contents, and the difference between MP2288 and MP2286 may in part be due to variability in intestinal fecal content. In separate studies, animals fasted for 24 h displayed much less uptake variability in the large intestines (data not shown). Urinary excretion was rapid for both compounds and not significantly different for MP2286 compared with MP2288 (84%±2.7% vs 77%±7.6%), and fecal excretion was low (6.2%±1.8% for MP2288 vs 7.2%±2.6% for MP2286).

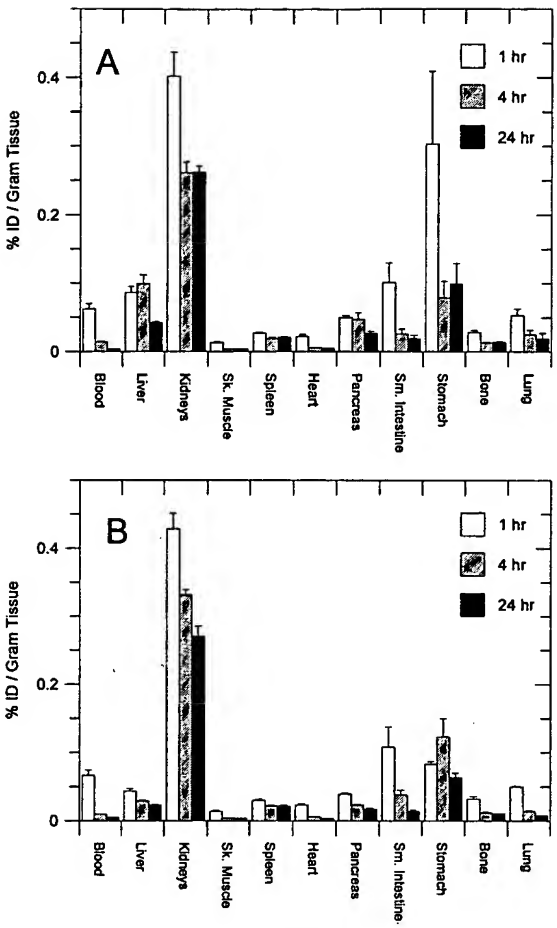

Fig. 4. Biodistribution of ^{111}In-MP2288 (A) and ^{111}In-MP2286 (B) in normal Sprague-Dawley rats at 1, 4, and 24 h post injection (see text for details). *Error bars* indicate standard error ($n = 3$)

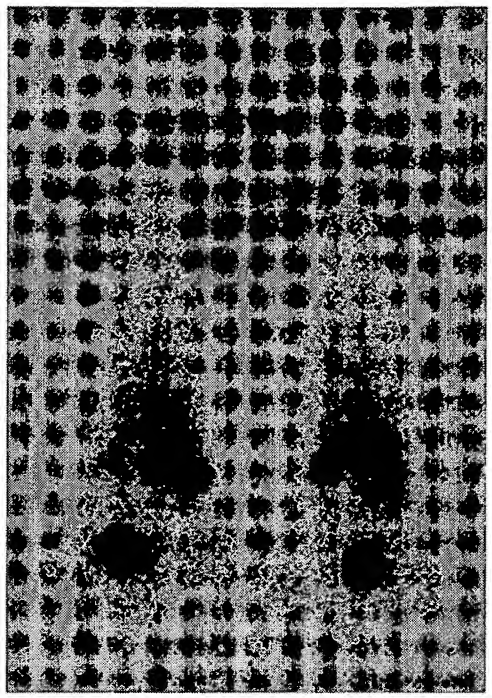

Fig. 5. Scintigrams of in vivo biodistribution of ^{111}In-MP2288 at high specific activity (1400 Ci/mmol) in two rats. Images were obtained at 90 min post injection. Strong labeling of the bladder and both kidneys is observed, suggesting rapid and predominantly renal clearance. Dorsal view

The overall recovery was nearly identical (93%±0.3% for MP2286 vs. 86%±5.3% for MP2288). Scintigraphic images of the biodistribution of MP2288 taken at 90 min p.i. in 24-h fasted rats are shown in Fig. 5 and underscore the rapid renal clearance and low-level uptake into most tissues.

To further evaluate the specificity of in vivo uptake of the labeled CCK-8 analogs into CCK-B receptor-expressing tissues of the gastrointestinal tract, a receptor blocking study was performed using 24-h fasted normal rats injected with ^{111}In-MP2288 radiolabeled at high or low specific activities (1400 Ci/mmol vs 0.5 Ci/mmol) to distinguish regions of nonspecific binding from regions which display specific CCK receptors. In a preliminary study with high specific activity ^{111}In-MP2288, fasted animals were imaged by scintigraphy at 15-min intervals over a 4-h time span. It was found that between 1 and 3 h a majority of the radioligand had cleared from most tissues but that significant uptake was still apparent in the gut region. We therefore sacrificed animals at 120 min post injection for evaluation. In order to map the entire region of the gastrointestinal tract for possible receptor expression, the whole gastrointestinal tract from the stomach to the distal large intestines was removed intact, photographed, and then scintigraphed (Fig. 6A and B). Figure 6B shows the ex vivo scintigraphy of the resected gastrointestinal tract from three rats. The stomachs displayed high uptake of radiolabeled peptide, corresponding to specific binding, since it was blocked in the presence of excess cold peptide (using low specific activity MP2288). The rest of the gastrointestinal tract did not show tracer uptake, except for an area which forms the boundaries of the duodenum and jejunum, where a strong, albeit apparently nonspecific uptake, was observed. Radioactivity associated with either the proximal or distal regions of the small intestines was not affected by the blocking dose (data not shown). In agreement with the above-described specific in vivo labeling of the stomach, in vitro receptor autoradiography revealed a high density of CCK-B receptors in the rat gastric mucosa, whereas ileum and colon did not show measurable amounts of CCK-B receptors, but only a moderate number of CCK-A receptors in the smooth muscles (Fig. 6C and D). The stomach tissue was examined further by determining total radioactivity of the intact organ, before and after removal of contents, with and without receptor blocking conditions. Specific in vivo binding to stomach tissue was only apparent after removal of

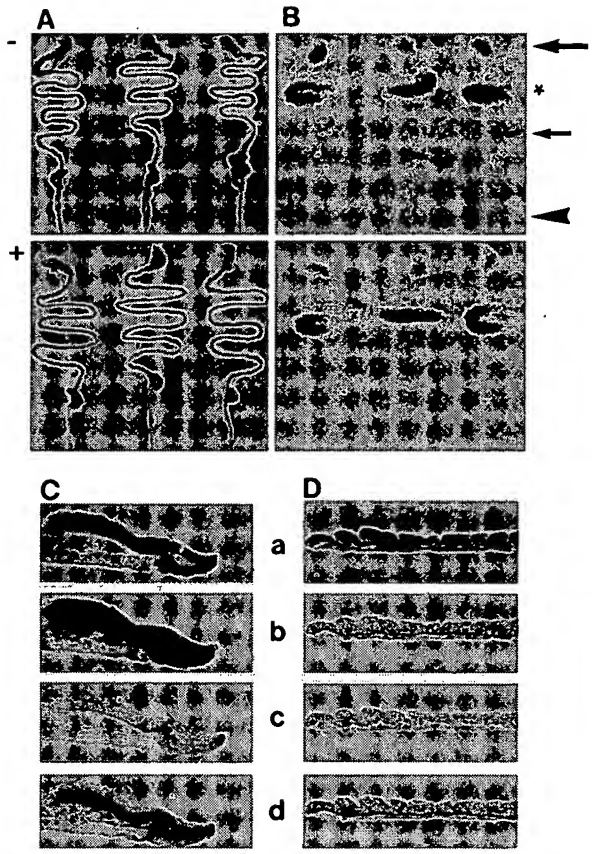

Fig. 6. A, B In vivo uptake of ^{111}In-MP2288 using high (–) and low (+) specific activity radiotracer (see Fig. 5 and text for additional details) measured in the corresponding excised gastrointestinal tracts of rats. **A** Photographs of the excised gastrointestinal tract. **B** Scintigrams of the excised gastrointestinal tract. *Large arrow*, stomach level; *small arrow*, ileum level; *arrowhead*, colon level; the star corresponds to a region of high nonspecific uptake (no blockade with excess cold peptide). The ^{111}In-MP2288 is only blocked with excess cold peptide at the stomach level, suggesting specific binding to CCK-B receptors. **C, D** In vitro CCK-receptor autoradiography of the rat stomach (**C**) and ileum (**D**). **a** Hematoxylin-eosin stained sections. **b** Autoradiographs showing total binding of ^{125}I-CCK, representing CCK-A and CCK-B receptors. **c** Autoradiographs showing nonspecific binding in the presence of 50 n*M* unlabeled sulfated CCK-8. **d** Autoradiographs showing non-specific binding in the presence of 50 n*M* unlabeled gastrin. The residual binding in **d** (in mucosa or muscles) represents CCK-A receptors only. The gastric mucosa has a high density of CCK-A and CCK-B receptors; the ileal mucosa has no CCK receptors, whereas the ileal muscles have only CCK-A receptors

the contents. Figure 7 shows that low specific activity MP2288 had a much reduced uptake compared with high specific activity MP2288, when measured in rat stomachs which were resected and then washed from their contents, confirming that specific CCK-B receptors can be specifically labeled in vivo with this CCK analog. The percent binding inhibition of low specific activity

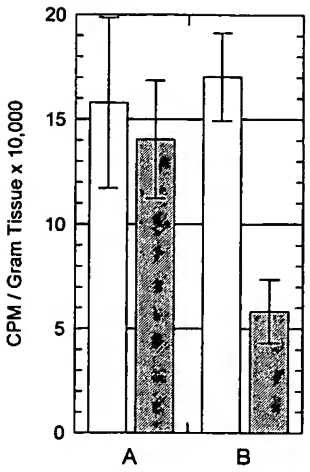

Fig. 7. In vivo uptake of high (*plain bars*) or low (*hatched bars*) specific activity radiotracer (1400 Ci/mmol and 0.5 Ci/mmol ^{111}In-MP2288 respectively) into stomach tissue before (**A**) and after (**B**) removal of contents. Radioactivity was measured by gamma counting and *error bars* indicated standard deviation (n = 3). An 1% injected dose corresponds to 425,000 cpm±4250 cpm. A strong blocking effect of excess cold peptide (= ^{111}In-MP2288 at low specific activity) is seen in the stomachs with their contents removed

MP2288 compared with high specific activity MP2288 amounted to 65.6% (Fig. 7).

Preliminary HPLC evaluation of excreted urine from rats injected i.v. with ^{111}In-MP2286 or ^{111}In-MP2288 showed that there was no intact peptide present 1 h after tracer injection (data not shown). A first attempt to examine the nature of this degradation and at which level it occurred was made by determining in vitro the stability of the two compounds in both rat and human plasma and urine. Very little degradation of either ^{111}In-MP2286 or ^{111}In-MP2288 was observed in rat or human plasma after incubation for 3 h at 37°C (>90% intact peptide). The compounds were also stable when incubated in the presence of the low molecular weight filtrates of rat or human urine filtered through an Amicon YM10 membrane (10,000-dalton cutoff) (data not shown) or in buffered saline for 3 h at 37°C (Fig. 8). This shows that the observed degradation is not due to ligand instability due to temperature, pH effects, or the presence of metal competitors. In contrast, in similar incubations, the two tested CCK derivatives were partly degraded in unfiltered urine or in urine protein (retained fraction of tenfold concentrated urine filtered on YM10 membrane, Fig. 8). Both compounds showed identical decay rates with more than 50% intact peptide present after 3 h. Both compounds yielded similar but not identical degradation products as determined by HPLC (Fig. 9). This provides further evidence that the observed products are DTPA-linked peptide degradation products and not the result of ^{111}In transchelation into high molecular weight urine factors.

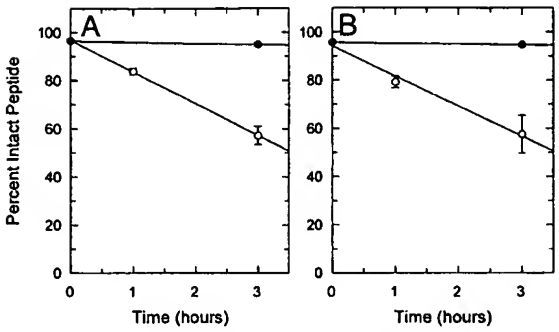

Fig. 8. In vitro degradation of ^{111}In-MP2288 (**A**) and ^{111}In-MP2286 (**B**) in human urine. Data obtained from integration of radiometric traces shown in Fig. 9 and graphed as percent intact peptide with respect to total eluted radioactivity. Saline controls incubated at 37°C for 3 h (●); and urine protein fraction incubated at 37°C for 3 h (○). *Error bars* indicate standard error ($n = 3$)

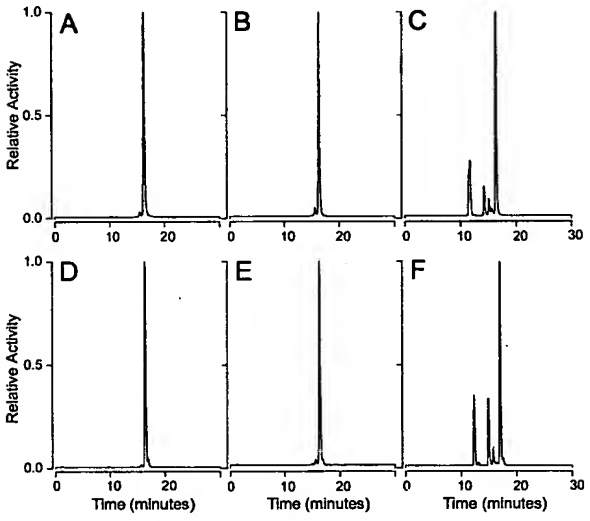

Fig. 9. In vitro degradation of ^{111}In-MP2288 (**A–C**) and ^{111}In-MP2286 (**D–F**) in human urine protein. Reverse-phase HPLC radiometric traces of control samples before incubation (**A, D**); saline control samples after 3 h incubation at 37°C (**B, E**); and samples incubated in saline with urine protein (**C, F**) after 3 h at 37°C (see methods for details). Secondary peaks in **C** and **F** indicate ^{111}In-DTPA-peptide-linked degradation products

Discussion

The present study describes for the first time peptidic CCK analogs potentially suitable for scintigraphic investigations. These new DTPA- or DOTA-linked CCK analogs are highly specific for the CCK-B receptor and exhibit a nanomolar binding affinity. The compounds which bind with high affinity have the DTPA or DOTA moiety coupled to the N-terminal end of the CCK octapeptide whereas those which have low or no measurable affinity have the chelator molecule at the C-terminal end. The high specificity towards CCK-B receptors is determined by the presence of nonsulfated tyrosine. Indeed, it is well established [13] that a sulfated tyrosine in position 27 of CCK-33 is necessary for CCK and short CCK derivatives to retain high affinity for both CCK-A and CCK-B receptors. Replacement of sulfated tyrosine with tyrosine results in compounds that bind selectively to the CCK-B receptor. Moreover, the high-affinity binding observed in vitro for the two best compounds, MP2286 and MP2288, is retained when they are chelated with ^{115}In. An IC_{50} value below 2 n*M* of this category of compounds is considerably better than the binding affinity of the ^{115}In-DTPA-octreotide to somatostatin receptors [14]. As expected, the DOTA-containing CCK octapeptide analog (MP2354) also binds with high affinity.

In addition to their selective and high-affinity binding characteristics, the two DTPA-CCK compounds also appear to be quite stable in human plasma. Other compounds tested, MP2247 and MP2290 for example, were much less stable (data not shown). Increased plasma stability is probably due, in part, to the substitution of the two methionines in these latter compounds for norleucine (present in MP2286 and MP2288) [15]. The instability of ^{111}In-labeled MP2288 and MP2286 found in urine and in a urine protein fraction may be related to the fact that the kidney is the major place of inactivation of CCK and gastrin [16]; this appears to be due to enzymatic degradation, although further studies are necessary to determine the nature of the degradation products observed in Fig. 9. It is not clear what effect urine stability would have on the overall imaging properties of these compounds.

The results of the in vivo rat biodistribution studies showed that both MP2286 and MP2288 clear rapidly by renal excretion, and display low uptake/retention in the main peripheral soft tissues. These properties are desirable for receptor-targeted imaging agents [1], particularly when the receptor targets are located in the thoracic area (i.e., for the visualization of SCLCs and MTCs). Generally, the two compounds distributed in a similar manner in vivo when compared directly with each other. Their general pattern of distribution is not basically different from that of ^{111}In-DTPA-D-Phe-octreotide [17], except that their kidney retention is much lower.

Blocking studies, where the uptake of radiolabeled CCK was determined in the presence and absence of an excess amount of unlabeled peptide, illustrate the in vivo specificity of the imaging molecules. The blocking effect of excess cold peptide was evaluated on the gastrin target tissues of normal fasted rats because a CCK-B-receptor expressing animal tumor model was not available. This study shows that MP2288 specifically binds in vivo to the CCK-B receptors present in normal CCK target tissues. Significant uptake of high specific activity ^{111}In-MP2288 into stomach tissue was observed, and a substantial blocking effect on the uptake was observed using low specific activity ^{111}In-MP2288. This is explained by the fact that the stomach, in rat and human, is one of the organs with the highest levels of CCK-B re-

ceptor expression [5]. Conversely, the rat ileum and colon, which express only CCK-A receptors, did not show uptake of the CCK radioligands in vivo. There was also a narrow region of uptake in the duodenal/jejunal region of the small intestines. This uptake was observed using both high and low specific activity peptides, suggesting low affinity or nonspecific binding only. No uptake of MP2288 was observed scintigraphically in brain tissue, which is rich in CCK receptors, because such peptide analogs cannot cross the intact blood-brain barrier, at difference to the nonpeptide CCK-analogs [18, 19]. Similar results were obtained previously in somatostatin receptor studies in rats where the in vivo uptake of ^{123}I-octreotide and ^{111}In-DTPA octreotide [14] in somatostatin receptor-expressing targets, such as adrenals, could be blocked with unlabeled octreotide. Evidence for the specificity of the visualization process is therefore clearly given by the present data, suggesting that DTPA-CCK radioligands can label physiological CCK-B receptors in vivo, even in the absence of an adequate tumor model.

The present study shows that CCK analogs N-terminally linked to chelators retain high affinity and high selectivity for CCK-B receptors. These derivatives also have considerable stability in plasma and are rapidly cleared from the circulation through the kidneys. Moreover, it has been shown previously that CCK-B receptors can be efficiently internalized into cells upon CCK ligand binding [20], a mechanism which is thought to represent the basis for the in vivo accumulation of peptide radioligands in the cells expressing the corresponding peptide receptors. All the above-mentioned characteristics make these DTPA- and DOTA-CCK compounds highly promising as radioligands for the in vivo targeting of human tumors expressing CCK-B receptors, such as MTCs, SCLCs, astrocytomas, and stromal ovarian cancers, as well as some gastrointestinal, pancreatic, and breast tumors [7, 21, 22]. According to published incidence figures [6, 7], CCK-B receptor scintigraphy should identify the great majority (>90%) of MTCs, considerably more than are presently detected with Octreoscan [1, 23]. It also may be possible to use a cocktail of radiolabeled DTPA-octreotide and DTPA-CCK to amplify the scintigraphic signal in MTCs, since both somatostatin and CCK-B receptors are expressed concomitantly in many of these tumors [24]. Finally, since DOTA-CCK analogs also bind with high affinity to CCK-B receptors, radiotherapy may be possible using an yttrium-90 labeled peptide [25, 26].

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Preclinical and Initial Clinical Evaluation of ^{111}In-Labeled Nonsulfated CCK_8 Analog: A Peptide for CCK-B Receptor-Targeted Scintigraphy and Radionuclide Therapy

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The presence of cholecystokinin (CCK)-B (gastrin) receptors has been shown in more than 90% of medullary thyroid cancers (MTCs) and in a high percentage of small cell lung cancers, stromal ovarium cancers and several other tumor types. **Methods:** The aim of this study was to evaluate in vitro and in vivo whether ^{111}In-labeled CCK-B receptor-specific CCK_8 analog [D-Asp^{26},Nle^{28,31}]CCK_{26-33} (D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2) is suitable for CCK-B receptor scintigraphy based on the finding that unlabeled nonsulfated diethylenetriamine pentaacidic acid [DTPA^0]CCK_8 and tetraazacyclododecanetetraacetic acid [DOTA^0]CCK_8 analogs show high and specific binding for CCK-B receptors in human tumors. Fifty percent inhibitory concentrations were in the low nanomolar range. **Results:** In vitro, [^{111}In-DOTA^0]CCK_8 showed specific internalization in CCK-B receptor-positive rat pancreatic tumor cells AR42J. Internalization of the analog appeared to be time and temperature dependent and receptor specific. From the data obtained with [^{111}In-DOTA^0]CCK_8 and ^{125}I-gastrin, the latter being a specific ligand for the CCK-B receptor, the rat pancreatic cell line CA20948 also appeared to be CCK-B receptor positive. This provides an in vitro and in vivo rat tumor model because this cell line can be grown to solid tumors in Lewis rats. In vivo biodistribution experiments in CA20948 tumor-bearing Lewis rats showed rapid clearance of [^{111}In-DOTA^0]CCK_8, and specific uptake was found in the CCK-B receptor-expressing stomach and tumor. Furthermore, comparing [^{111}In-DOTA^0]CCK_8 with the radioiodinated nonsulfated CCK_{10} analog (D-Tyr-Gly-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2), both ligands having high affinity for the CCK-B receptor, tumor-to-blood ratios were significantly higher for [^{111}In-DOTA^0]CCK_8 than for ^{125}I-CCK_{10}, analogous to the findings with radioiodinated and ^{111}In-labeled octreotide. The study in humans with [^{111}In-DTPA^0]CCK_8 showed receptor-specific uptake in the CCK-B receptor-positive stomach and in metastases in the neck region up to 48 h after injection. **Conclusion:** [^{111}In-DOTA^0]CCK_8 is most promising for scintigraphy and, after coupling to therapeutic radionuclides, for radionuclide therapy of human CCK-B receptor-positive tumors such as MTC and small cell lung cancer.

Key Words: cholecystokenin receptors; [DOTA^0]CCK_8; [DTPA^0]CCK_8; ^{111}In; tumor scintigraphy; radionuclide therapy; medullary thyroid cancers

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Radiolabeled tumor receptor-binding peptides can be used for in vivo scintigraphic imaging. The peptides most widely used now are stable somatostatin analogs that bind to their receptors on tumors of neuroendocrine origin (*1*). An example is the octapeptide [^{111}In-DTPA^0]octreotide, consisting of octreotide and the chelator diethylenetriamine pentaacetic acid (DTPA), enabling instant radiolabeling with a radiometal such as ^{111}In. We have described its use for scintigraphic imaging of somatostatin receptor-positive lesions, such as gastroenteropancreatic neuroendocrine tumors, neuroblastoma, pheochromocytoma, breast cancer, Hodgkin's lymphoma and small cell lung cancer (*2,3*). However, unlike in other neuroendocrine tumors, somatostatin receptor expression is rather low in medullary thyroid cancer (MTC) and is completely absent in clinically aggressive forms of the disease (*4,5*). Recently, the presence of cholecystokinin (CCK)-B (gastrin) receptors was shown in more than 90% of MTCs, and the presence of these receptors was shown in a high percentage of small cell lung cancers, stromal ovarium cancers, astrocytomas and several other tumor types (*6*). On the basis of these findings, Behr et al. (*7*) evaluated the suitability of radioiodinated gastrin, a specific ligand for the CCK-B receptor, for targeting CCK-B receptor-expressing tumors in vivo. Their data suggest that gastrin and its analogs may represent a useful new class of receptor-binding peptides for diagnosis and therapy of a variety of tumor types, including MTC. Furthermore, Reubi et al. (*8*) developed DTPA-conjugated CCK-B receptor-binding CCK analogs, evaluated their receptor-binding characteristics and obtained initial preclinical biodistribution data in nontumor-bearing rats. For the tetraazacyclododecanetetraacetic acid (DOTA) counterpart of the most

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promising analog, a high CCK-B receptor affinity was found. They concluded that CCK analogs are promising for human CCK-B receptor scintigraphy as well (*8*).

A new application is the use of radiolabeled peptides for radionuclide therapy. Promising results in tumor growth inhibition using [^{111}In-DTPA^0]octreotide have been reported in humans (*9*). Besides Auger electron emitters, such as ^{111}In, β^- particle emitters, such as ^{90}Y, may appear suitable for this purpose. The ^{90}Y-DTPA complex is unstable, resulting in hematopoietic toxicity in vivo. Therefore, the DOTA chelator, which forms stable complexes with ^{90}Y and ^{111}In, was coupled to CCK_8, enabling future radionuclide therapy of MTC.

The success of the therapeutic strategy relies on the amount of radioligand that can be concentrated within tumor cells and thus the rates of internalization, degradation and recycling of both ligand and receptor. Binding of several peptide hormones to specific surface receptors is generally followed by internalization of the ligand-receptor complex. Williams et al. (*10*) and Svoboda et al. (*11*) have reported internalization of unchelated CCK. The internalization process of this peptide appears to be CCK receptor specific and temperature dependent. One aim of this study was to evaluate internalization of [^{111}In-DOTA^0]CCK_8 in rat pancreatic tumor cells in vitro.

Biodistribution and tumor visualization also were investigated in vivo in tumor-bearing animals. Biodistribution of [^{111}In-DOTA^0]CCK_8 was compared with that of ^{125}I-CCK_{10} analog (*8*), analogous to our studies using radioiodinated and ^{111}In-labeled octreotide, to compare the differences in biodistribution and cellular retention of the peptides radiolabeled with either a residualizing radiolabel like ^{111}In or with a nonresidualizing radiolabel like ^{125}I. We performed toxicity studies with unlabeled [DOTA^0]CCK_8 and [DTPA^0]CCK_8 in rats and mice, and, thereafter, an initial evaluation of [^{111}In-DTPA^0]CCK_8 in humans was performed.

MATERIALS AND METHODS

Compounds

^{125}I-gastrin (74×10^{12} Bq/mmol) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Synthetic gastrin was from Bachem (Bubendorf, Switzerland). This is a heptadecapeptide, specific for the CCK-B/gastrin receptor with an affinity constant in the subnanomolar range, whereas its affinity for the CCK-A receptor is lower by more than four orders of magnitude. Mallinckrodt, Inc. (Petten, The Netherlands) provided $^{111}InCl_3$. DOTA and DTPA analogs of CCK_8 (D-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2) were synthesized according to previously described methods (*8*). ^{125}I-CCK_{10} (D-Tyr-Gly-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2) was synthesized by Chiron (*8*). ^{111}In-labeling of the DTPA and DOTA analogs was as described for [DTPA^0]octreotide (*2*) and [DOTA^0,Tyr^3]octreotide (*12*), respectively. ^{125}I-labeling of ^{125}I-CCK_{10} was performed as described for [Tyr^3]octreotide (*13*).

Internalization

AR42J cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY), CA20948 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) and ARO cells were grown in DMEM/F12 medium (Gibco). For all cell lines, medium was supplemented with 2 mmol/L glutamine and 10% fetal calf serum (Gibco). Before the experiment, subconfluent cell cultures were transferred to six-well plates.

The binding of the radiolabeled peptides to tumor cells and subsequent internalization were studied as described (*14*). Cells were washed and incubation was started by addition of 1 mL internalization medium per well (culture medium without fetal calf serum but with 1% bovine serum albumin [Sigma, St. Louis, MO]) with about 80 kBq of radiotracer. Peptide concentration range in the different experiments was 0.1–1 μmol/L. Cells were incubated at 37°C for indicated periods of time. To determine nonspecific internalization, cells were incubated with an excess unlabeled peptide (0.1 μmol/L). Cellular uptake was stopped by removing medium from the cells and washing with 2 mL ice-cold phosphate-buffered saline. To discriminate between internalized and noninternalized (surface-bound) radiopharmaceutical, intact cells were incubated with 1 mL 20 mmol/L sodium acetate. The internalized and noninternalized fractions were determined by measuring radioactivity. The internalized fraction was expressed as percentage of the applied dose per milligram cellular protein. The latter was determined using a commercially available kit (Bio-Rad, Veenendaal, The Netherlands).

Data are expressed as mean ± SD for incubations assayed in triplicate, with each experiment performed at least three times.

In Vivo Tissue Distribution

Animal experiments were performed in compliance with the regulations of this institution and with generally accepted guidelines governing such work. Male Lewis rats (200–250 g), bearing the CA20948 pancreatic tumor, were used in the experiments. Rats were injected under ether anesthesia with 3 MBq (0.5 μg) ^{111}In-labeled peptide in 200 μL saline into the dorsal vein of the penis. To determine nonspecific binding of the radiopharmaceutical, a separate group of rats was coinjected intravenously with 0.1 mg [DOTA^0]CCK_8. At the indicated time points, rats were killed under ether anesthesia. Organs and blood were collected, and the radioactivity in these samples was determined. Results are expressed as mean ± SD of at least six rats per group.

Toxicity Study of Unlabeled [DOTA^0]CCK_8 and [DTPA^0]CCK_8

On experimental day 0, eight treatment groups of five Wistar rats and five BALB/c mice were injected intravenously with saline (0 times), 10 times, 100 times or 1000 times the human concentration of 15 μg/75 kg of [DOTA^0]CCK_8 or [DTPA^0]CCK_8, respectively. All test solutions were injected through the penis vein at a rate not exceeding 1 mL/min. Until 24 h after injection the animals were monitored for changes in behavior (eating, sleeping, motion, posture) and signs of toxicosis. Any reaction to the treatment was recorded. All rats were killed by ether narcosis 24 h after injection and subjected to detailed macroscopic postmortem examination. After examination of the external surfaces, the chest and abdomen were opened by midline incision. Thoracic and abdominal viscera were examined for abnormalities. Aberrations were recorded. Organs were investigated macroscopically (e.g., for bleedings). Liver, kidneys, stomach, spleen, lungs and intestines were isolated and fixed in 4% buffered formalin. The organs were sectioned, stained and evaluated by light microscopy.

Initial Human Evaluation of [^{111}In-DTPA^0]CCK$_8$

A 45-y-old woman with local recurrence of MTC was injected with 222 MBq ^{111}In-labeled [DTPA^0]CCK$_8$ (10 μg peptide). Scans were acquired 4, 24 and 48 h after injection as described (*2,3*). Urine was collected during the first 24 h after injection.

RESULTS

Radiolabeling

^{111}In-labeling efficiency of the different peptides and radioiodination efficiency of ^{125}I-CCK$_{10}$ ranged from 95% to 100%. Radiochemical purity always exceeded 90%.

In Vitro Internalization Studies

Figure 1A shows the time- and temperature-dependent internalization of [^{111}In-DOTA^0]CCK$_8$ in the CCK-B receptor-positive AR42J rat pancreatic tumor cells and in the CCK-B receptor-negative ARO human anaplastic thyroid tumor cell line. Internalization was dose dependent and was reduced in the presence of increasing concentrations of unlabeled peptide, indicating that this process is receptor specific. Furthermore, it was reduced at 6°C versus 37°C, indicating temperature dependence, and increased over time. The acid-removable ("surface-bound") uptake was about 5%–10% of the internalized fraction (not shown). The ARO cells were used as negative controls. In these cells, internalization of [^{111}In-DOTA^0]CCK$_8$ was indeed low and showed no specific temperature-dependent accumulation. Figure 1B shows the inhibitory effect in AR42J cells of excess medium gastrin concentrations on internalization of ^{125}I-gastrin, a specific ligand with high affinity for CCK-B receptors, indicating specific internalization in these AR42J cells.

Figure 2A shows the time- and temperature-dependent internalization of [^{111}In-DOTA^0]CCK$_8$ in the CA20948 rat pancreatic tumor cells. Internalization was reduced in the presence of increasing concentrations of unlabeled peptide. Furthermore, it was reduced at 6°C, indicating temperature dependence, and increased over time. The acid-removable (surface-bound) uptake was about 5%–10% of the internalized fraction (not shown). Figure 2B shows internalization of ^{125}I-gastrin in the presence or absence of excess medium gastrin concentrations. In the presence of unlabeled gastrin, internalization was significantly reduced, indicating the presence of CCK-B receptors on the CA20948 rat pancreatic tumor cells.

Tissue Distribution in Rats

Table 1 compares the radioactivity in several organs after injection of [^{111}In-DOTA^0]CCK$_8$ and [^{111}In-DTPA^0]CCK$_8$. A rapid clearance from the blood through renal excretion was found for both radiolabeled compounds, with a pronounced uptake in the kidneys. Receptor-negative organ radioactivity paralleled blood clearance, resulting in a very low background radioactivity 24 h after injection. Comparison of biodistribution data and tumor uptake of [^{111}In-DOTA^0]CCK$_8$ and [^{111}In-DTPA^0]CCK$_8$ showed no major differences.

Figure 3 shows that uptake of [^{111}In-DOTA^0]CCK$_8$ in stomach and tumor, 4 h after injection, was at least partially receptor specific because uptake was significantly reduced after coinjection with 0.1 mg unlabeled peptide. Uptake in the other organs and radioactivity in blood did not change significantly after coinjection of unlabeled peptide (not shown).

Figure 4 shows a comparison of tissue radioactivity after injection of [^{111}In-DOTA^0]CCK$_8$ and ^{125}I-CCK$_{10}$, 1 and 4 h after injection, in several rat organs. Clearance from the blood was slower for ^{125}I-CCK$_{10}$ than for [^{111}In-DOTA^0]CCK$_8$, and uptake in receptor-negative organs, of which the liver is shown as an example, was higher for ^{125}I-CCK$_{10}$ than for [^{111}In-DOTA^0]CCK$_8$. A higher uptake of ^{125}I-CCK$_{10}$ than of [^{111}In-DOTA^0]CCK$_8$ was also found in the receptor-positive stomach, which seemed to be favorable for ^{125}I-CCK$_{10}$. However, the stomach-to-blood ratio, which represents the target-to-background ratio, was significantly lower for ^{125}I-CCK$_{10}$ than for [^{111}In-DOTA^0]CCK$_8$ at 4 h after injection.

Figure 5 shows a gamma camera scan, made 20 min after injection, of two tumor-bearing rats and a control rat,

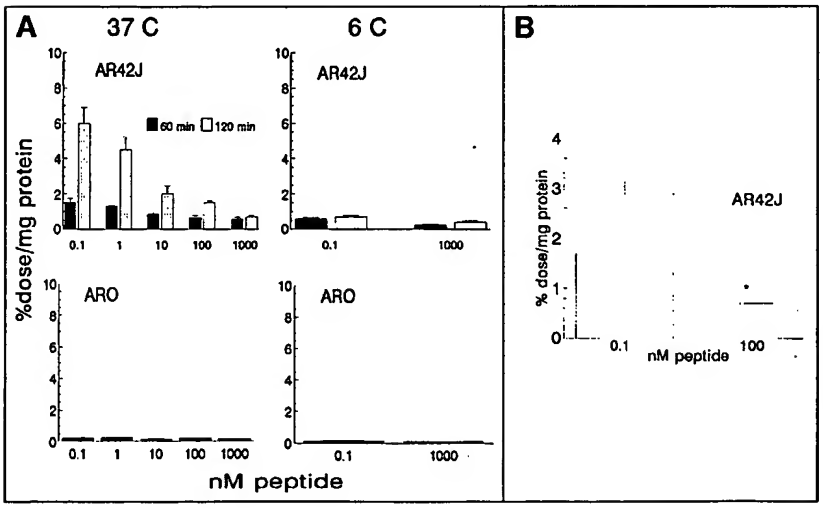

FIGURE 1. (A) Internalization of [^{111}In-DOTA^0]CCK$_8$ in CCK-B receptor-positive AR42J rat pancreatic tumor cells and in CCK-B receptor-negative ARO human anaplastic thyroid tumor cell line. Data are expressed as percentage added dose/mg protein (mean ± SD). (B) Internalization, after 60-min incubation, of ^{125}I-gastrin, specific ligand with high affinity for CCK-B receptor, in AR42J cells. Data are expressed as percentage added dose/mg protein (mean ± SD).

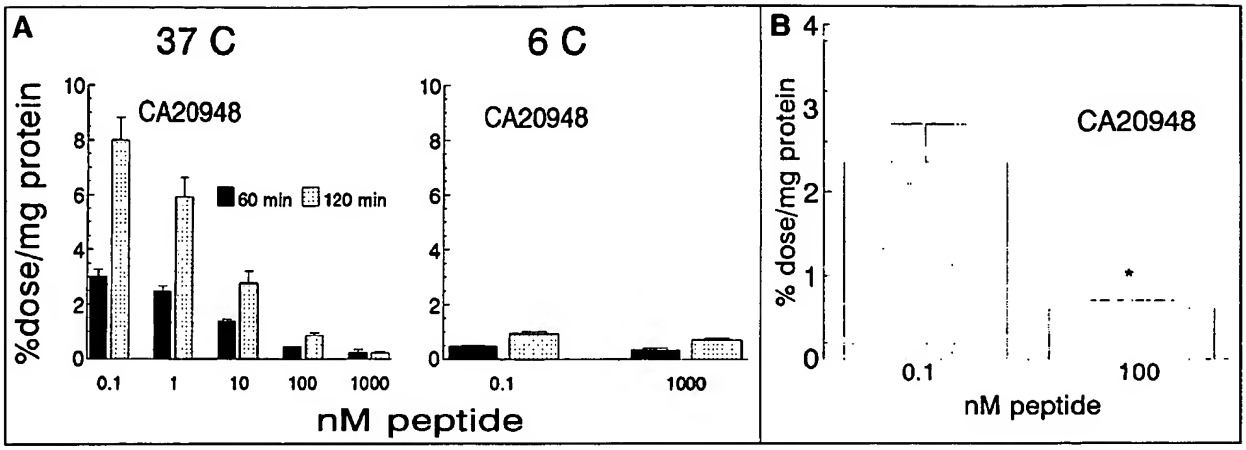

FIGURE 2. (A) Internalization of [^{111}In-DOTA^0]CCK$_8$ in CCK-B receptor-positive CA20948 rat pancreatic tumor cells. Data are expressed as percentage added dose/mg protein (mean ± SD). (B) Internalization, after 60-min incubation, of ^{125}I-gastrin, specific ligand with high affinity for CCK-B receptor, in CA20948 cells. Data are expressed as percentage added dose/mg protein (mean ± SD).

injected with [^{111}In-DOTA^0]CCK$_8$ without or with excess unlabeled peptide or with ^{125}I-CCK$_{10}$. The radioiodinated analog had a different distribution pattern with much higher liver uptake than did [^{111}In-DOTA^0]CCK$_8$, which is cleared through the kidneys. Furthermore, the CCK-B receptor-positive tumor was clearly visualized in the left rat but was not detected after coinjection with unlabeled peptide, as shown in the middle rat.

Toxicity Study

No abnormalities were found in animal behavior during the 24 h after injection. No macroscopic pathology was observed. Microscopic examination of representative tissue sections from rats treated with [DOTA^0]CCK$_8$ revealed no abnormalities that could be attributed to the treatment.

TABLE 1
Radioactivity in Organs and Tumor of CA20948 Tumor-Bearing Rats 1, 4 and 24 Hours After Administration of [^{111}In-DOTA^0]CCK$_8$ or 24 Hours After Administration of [^{111}In-DTPA^0]CCK$_8$

	DOTA* time administration			DTPA* time administration
Tissue	1 h	4 h	24 h	24 h
Blood	0.099 (0.010)	0.018 (0.002)	0.005 (0.001)	0.005 (0.001)
Spleen	0.041 (0.004)	0.034 (0.004)	0.033 (0.003)	0.023 (0.003)
Pancreas	0.037 (0.002)	0.013 (0.001)	0.011 (0.000)	0.012 (0.000)
Adrenals	0.086 (0.016)	0.017 (0.002)	0.019 (0.000)	0.018 (0.000)
Kidneys	0.706 (0.11)	0.439 (0.061)	0.379 (0.050)	0.322 (0.03)
Liver	0.040 (0.011)	0.027 (0.004)	0.026 (0.005)	0.045 (0.007)
Stomach	0.08 (0.02)	0.044 (0.004)	0.028 (0.006)	0.035 (0.007)
Colon	0.041 (0.013)	0.013 (0.003)	0.037 (0.008)	0.01 (0.001)
Muscle	0.012 (0.001)	0.003 (0.000)	0.003 (0.000)	0.003 (0.000)
Femur	0.035 (0.002)	0.014 (0.001)	0.012 (0.001)	0.011 (0.001)
Pituitary	0.012 (0.002)	0.004 (0.001)	0.003 (0.000)	
Tumor	0.160 (0.021)	0.130 (0.020)	0.082 (0.011)	0.094 (0.02)

*Tissue radioactivity is expressed as percentage injected dose/g tissue (mean ± SD). For each group, n ≥ 4.

DOTA = tetraazacyclododecanetetraacetic acid CCK$_8$ analog; DTPA = [^{111}In]diethylenetriaminepentaacetic acid CCK$_8$ analog.

Initial Human Evaluation of [^{111}In-DTPA^0]CCK$_8$

Intravenous injection of 10 μg peptide was well tolerated by the patient, with no adverse reactions. Figure 6 shows a

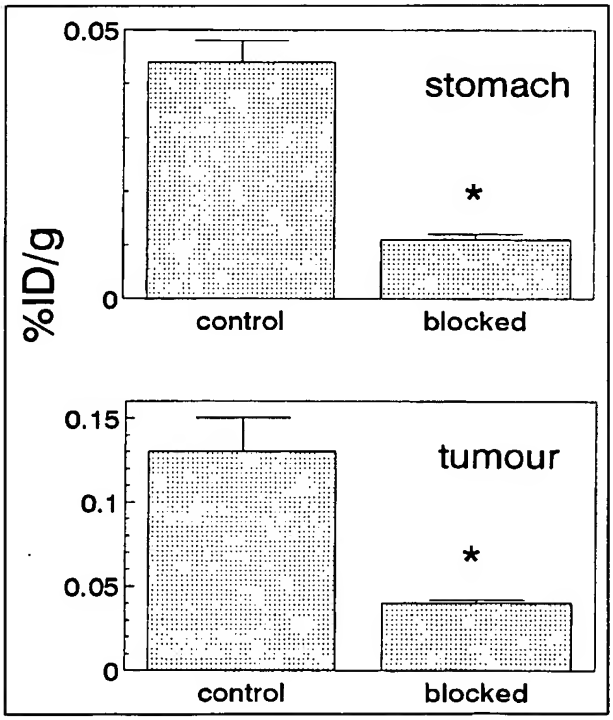

FIGURE 3. Uptake in rat stomach and CA20948 tumor, 4 h after injection of [^{111}In-DOTA^0]CCK$_8$, with or without coinjection with 100 μg unlabeled peptide. %ID/g = percentage injected dose/g tissue (mean ± SD).

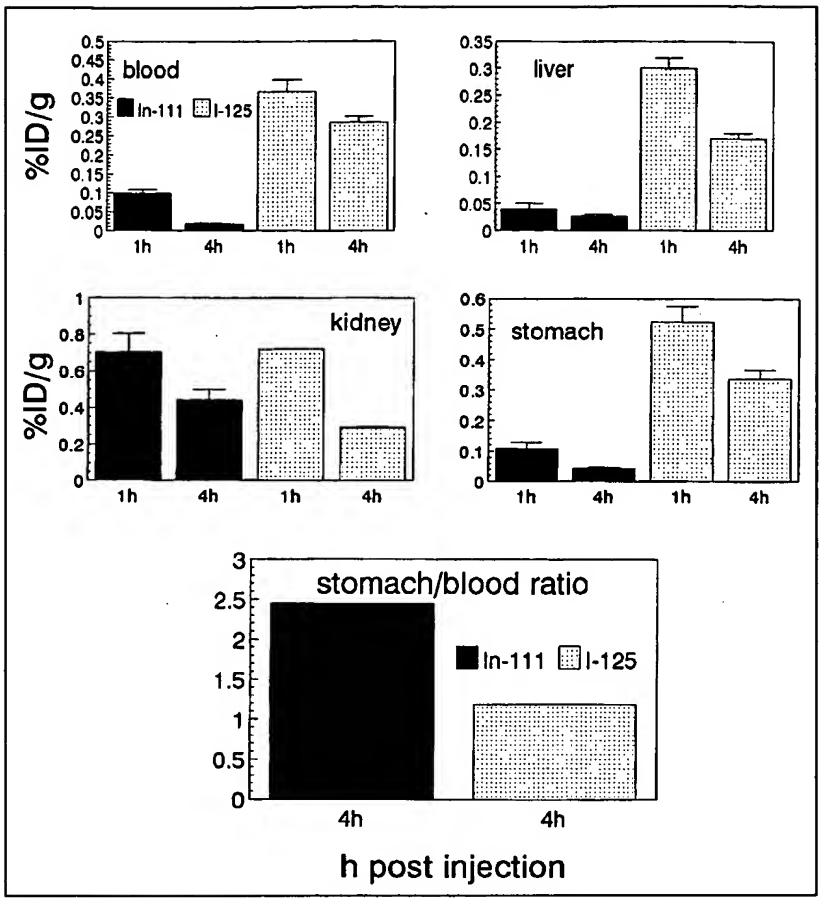

FIGURE 4. Radioactivity in several rat organs, 1 and 4 h after injection of ^{125}I-CCK_{10} and [^{111}In-DOTA^0]CCK_8. %ID/g = percentage injected dose/g tissue (mean ± SD).

scan of the upper abdomen and neck, taken 48 h after injection. As in the preclinical studies in rats, receptor-specific uptake was seen in the CCK-B receptor-expressing fundus of the stomach and in metastases in the neck region. Cumulative excretion of radioactivity in the urine was more than 90% of the dose at 24 h after injection.

DISCUSSION

Autoradiographic studies by Reubi et al. (*6*) revealed the presence of CCK-B receptors in more than 90% of MTCs and in a high percentage of other tumors. Therefore, CCK-B receptors appeared to represent a new and promising target for radiolabeled peptides for tumor scintigraphy and radionuclide therapy. Behr et al. (*7*) evaluated the suitability of radioiodinated gastrin, a specific ligand for the CCK-B receptor, for targeting CCK-B receptor-expressing tumors in vivo. Reubi et al. (*8*) also developed DOTA- and DTPA-conjugated CCK-B receptor-binding CCK analogs, evaluated their receptor binding characteristics and obtained initial preclinical biodistribution data using [DTPA^0]CCK_8 in nontumor-bearing control rats. They concluded that the peptide analogs used were promising for human CCK-B receptor scintigraphy and radionuclide therapy.

For these studies, we chose nonsulfated octapeptide CCK_8, derivatized with either DOTA or DTPA, because this peptide has shown high affinity for CCK-B receptors but low affinity for CCK-A receptors (*8*), the latter in contrast to sulfated analogs that bear a sulfate ester attached to the Tyr moiety. CCK-A receptors are expressed at a much higher density in normal tissues, especially in the abdomen. The selective CCK-B receptor affinity of the chosen nonsulfated analog will result therefore in a favorable low background radioactivity during scanning.

For the success of radionuclide therapy, it is important that the tumor cells internalize the radiopharmaceutical after binding to the receptor. We demonstrated receptor-specific, time- and temperature-dependent internalization of [^{111}In-DOTA^0]CCK_8 in AR42J cells, indicating that the DOTA group does not prevent the CCK analog from internalization. In this study we also demonstrated receptor-specific internalization of ^{125}I-gastrin and [^{111}In-DOTA^0]CCK_8 in CA20948 cells in culture, consistent with the presence of CCK-B receptors in these tumor cells. This provides a tumor model for research on CCK analogs in vivo because these cells can also be grown to solid tumors in vivo in Lewis rats.

For [^{111}In-DTPA^0]octreotide, the internalization pathway proceeds through endosomes fusing with lysosomes where degradation of the radiolabeled peptide occurs. After dissociation of the ligand, the receptor protein may return to the plasma membrane, whereas ^{111}In-DTPA-containing degrada-

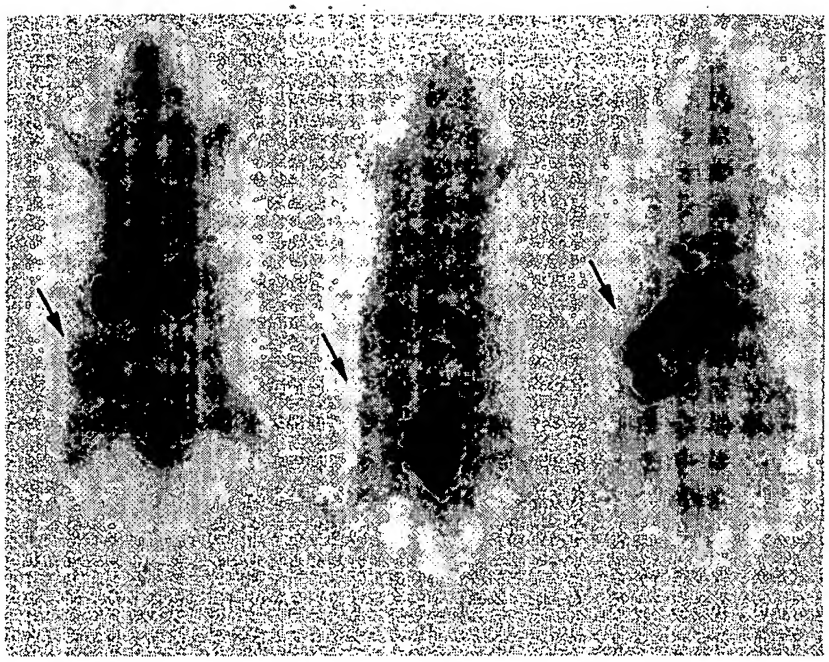

FIGURE 5. Scan, made 20 min after injection, of two tumor-bearing rats (left and middle) and control rat (right), injected with [^{111}In-DOTA^0]CCK$_8$ (left), [^{111}In-DOTA^0]CCK$_8$ in the presence of excess unlabeled peptide (middle) or ^{125}I-CCK$_{10}$ (right). Left and middle arrows indicate tumor uptake; right arrow indicates liver uptake.

tion products are retained in the lysosomes, causing the long retention time of radioactivity in receptor-positive cells (*15*). The assumption that this intracellular route holds also for CCK-B receptor-specific peptides is in accordance with the long retention time of the radiolabel shown in the human study, where CCK-B receptors in stomach and metastases were visualized up to 48 h after injection.

Uptake of the ^{111}In-labeled peptide in CCK-B receptor-expressing tissues (stomach and CA20948 tumor) in vivo in rats was also found to be specific because uptake was reduced significantly in the presence of excess unlabeled hormone. Furthermore, in agreement with the assumption that unsulfated CCK analogs will result in low background radioactivity, uptake in receptor-negative organs was indeed low, which is favorable during scintigraphy and radionuclide therapy. These findings also showed that the tumor-to-background ratio for [^{111}In-DOTA^0]CCK$_8$ already was significantly higher than that for ^{125}I-CCK$_{10}$ at 4 h after injection, analogous to the findings with octreotide (*16*). Because we know that the intracellular retention of radioactivity is much longer for the residualizing ^{111}In-chelator peptides than for radioiodinated peptides (*16*), this tumor-to-background ratio will be increasingly favorable for the ^{111}In-labeled peptide. Furthermore, the high liver uptake found with the radioiodinated compound is very unfavorable during scintigraphy of tumors in the upper abdomen. Behr et al. (*7*), using ^{131}I-gastrin, found that a tumor-to-blood ratio of about 5 could be reached in nude mice bearing the human MTC tumor. In this study, a tumor-to-blood ratio of 16 was reached 24 h after injection, showing that residualizing radiolabels, such as the radiometal ^{111}In, have advantages over ^{131}I. This was also shown in human studies. After injection of ^{131}I-gastrin, good tumor-to-nontumor ratios were maintained for about 3 h (*7*), whereas in this study, using the ^{111}In-labeled peptide, the receptor-positive stomach (*17*) and tumor metastases were clearly visualized even 48 h after injection.

Despite the fact that the excretion of [^{111}In-DOTA^0]CCK$_8$ was through renal excretion, the uptake of radioactivity in the kidneys was about one order of magnitude lower than that of [^{111}In-DTPA]octreotide, a DTPA-coupled peptide also excreted in the urine. [^{111}In-DOTA^0]CCK$_8$ contains two

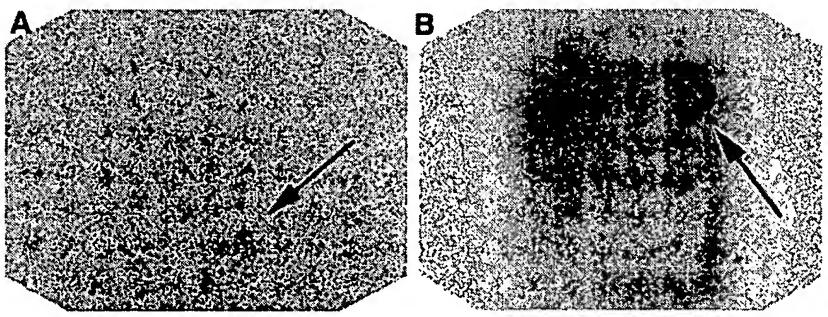

FIGURE 6. Visualization of CCK-B receptors in 45-y-old woman with MTC after intravenous administration of 222 MBq [^{111}In-DTPA^0]CCK$_8$ (10 μg peptide). Scans at 48 h after injection show uptake in lymph node metastases in neck region (A, arrow) and in receptor-positive stomach (B, arrow).

Asp moieties, giving an anionic charge to the molecule, whereas octreotide contains the positively charged Lys moiety. Negatively charged peptides apparently have a lower renal uptake in the environment of negatively charged membranes of tubular cells than neutral or cationic ones, consistent with the finding that positively charged amino acids can block peptide reabsorption by binding to the negatively charged membranes of the tubular cells (*18*). This low kidney uptake is very favorable during scintigraphy in the perirenal region; during radionuclide therapy studies, it will prevent renal radiotoxicity.

CONCLUSION

[^{111}In-DOTA^0]CCK$_8$ is most promising for scintigraphy and, after coupling to therapeutic radionuclides, for radionuclide therapy of CCK-B receptor-positive tumors, such as MTC and small cell lung cancer.

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